The role of interferon regulatory factors in LGTV infection

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aus Cottbus

1. Referent: Professor Dr. Martin Korte
2. Referentin: Professorin Dr. Andrea Kröger
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Zegenhagen, L., Weber E., Nair S., Lienenklaus S., Overby A., Kröger A.: The impact of Interferon regulatory factors on Type I IFN induction in Langat virus infection. (Poster). PhD Symposium, Braunschweig (2015).

Abstract

The activation of the innate immune response protects the host against viral infections. Innate recognition of viral signatures by pattern recognition receptors (PRRs) triggers the induction of type I Interferons (IFN) and results in a broad antiviral response. Here we show that various signaling components of the type I IFN pathway play a role in host defense against Langat virus (LGTV), a *Flavivirus* of the tick borne encephalitis virus (TBEV) serocomplex. IFN-β promoter stimulator-1 (IPS-1) and myeloid differentiation primary response 88 (Myd88) which are adaptor molecules for PRRs like retinoic acid induced gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and Toll like receptors (TLRs) promote survival in systemic LGTV infection. Further regulatory elements of the type I IFN pathway such as IFN- regulatory factor (IRF) -3 and IRF-7 mediate local protection against viral replication and dissemination, even though they are dispensable for survival. Especially IRF-7 limits replication and spread of LGTV in the periphery and plays a protective role in the central nervous system (CNS). The absence of IRF-7 in the brain was associated with higher local inflammation and a shift in tissue tropism. Neurons are already known to be highly susceptible to LGTV infection, but here it was shown that especially astrocytes rely on IRF-7 for protection against LGTV infection and propagation.

IRF-7 normally provides a positive feedback loop to amplify the type I IFN response. The lack of IRF-7 was to date associated with decreased type I IFN production. Here it was shown that astrocytes are able to provide a potent local type I IFN response by an IRF-7 independent mechanism. This thesis provides new insights in antiviral defenses in the absence of IRF-7, specifically in the CNS.

Contents

1. Introduction

Infectious diseases are a major risk of public health and much effort has been invested into the development of therapeutics and precautionary measures [1]. New or reemerging pathogens provide new challenges and the development of therapeutics is comparably slow and limited at best [1–3]. Therefore it is important to increase the understanding about the most effective defense system against pathogens namely the immune system of the host. Profound knowledge of the immune response might help to consider new therapeutic options and provide alternative approaches to fight against infections more effectively [4–6]. The immune system itself is an evolutionary shaped and refined program which is distinguished roughly into innate and adaptive immunity [7].

1.1. Innate Immunity

The innate immune system is an ancient part of the immune system that allows unspecific but fast and efficient antiviral responses [7]. This is in contrast to adaptive immunity which shapes immunological memory upon pathogenic encounter and elicits specific immune responses upon infection with harmful agents [7].

For the innate immune response distinct cell populations such as monocytes, macrophages, Dendritic cells (DCs) granulocytes and natural killer cells (NK cells) enable the organism to mount fast responses against invading pathogens as a first line of defense [8–10]. Whereas some cells show phagocytic activity, others are able to neutralize pathogens by neutrophil extracellular trap (NETs) formation [8][10]. However, cells of the innate immune system also contribute to the adaptive response [9]. DCs, neutrophils and macrophages for instance have a role in antigen presentation or secretion of inflammatory cytokines that induce T and B cell migration to the site of inflammation [8][11].

The complement system is another part of the innate immune response and consists of soluble proteins which are able to bind pathogens. [12]. Binding mediates direct pathogen killing by formation of pores in the cell membrane. Some components of the complement system also act as chemokines and therefore activate and modulate the immune response [13,14].

Cytokines and chemokines can also be released by immune cells as part of the adaptive as well as the innate immune response to mediate inflammatory and anti-inflammatory effects [15,16]. This initiates cell migration, cell differentiation as well as cell communication [17]. Similarly, small peptides called defensins support innate immunity by

unspecific antimicrobial effects within the cell [18]. The innate immunity therefore commands different weapons to fight against invading pathogens.

1.1.1. Pathogen recognition by the innate immune system

Pathogens such as bacteria, viruses or parasites can trigger cell intrinsic signaling pathways which lead to the production of defense molecules and the regulation of important cell functions like proliferation and survival [19–21]. Pathogen associated molecular patterns (PAMPS) like viral single stranded RNA (ssRNA), can be recognized by pathogen recognition receptors (PRRs) such as retinoic acid–inducible gene-I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) [22].

TLRs and CLRs function as membrane bound receptors, whereas Retinoic acid-inducible gene-I (RIG-I) and Melanoma differentiation-associated protein 5 (MDA5) act as cytosolic sensor proteins [23]. RIG-I and MDA-5 oligomerize upon activation and signal downstream to adapter protein interferon beta (IFN-β) promotor stimulator-1 (IPS-1), also known as CARDIF or MAVS, which is located on the mitochondrial membrane [24]. IPS-1 initiates a succession of phosphorylation steps with the involvement of different kinases such as TNF receptor associated factor 3 (TRAF3), TRAF family member-associated NF-kappa-B activator (TANK), TANK binding kinase-1 (TBK-1) or IkB kinase ε (IKKε) [25]. These kinases activate interferon regulatory factor-3 (IRF-3) which homodimerizes and translocates into the nucleus to induce transcription of IFN-β and IFN-α4 genes by the binding of IRF-3 specific IFN stimulated response element (ISRE) motifs [26,27]. Interferons are produced and secreted by the cell and bind to IFN-α/β receptor (IFNAR) in a paracrine or autocrine manner [20]. This leads to the induction of the Jak/Stat pathway [28]. Latent Signal transducers and activators of transcription 1 (Stat1) and Stat2 are phosphorylated by Janus kinases, dimerize and form the IFN-stimulated gene factor 3 (ISGF3) complex together with IRF-9 [29]. This complex induces the transcription of a broad range of IFN stimulated genes (ISGS), like 2'-5'-oligoadenylate synthetase (OAS), Viperin, Caspase-1 or IRF-7 which mediate multiple antiviral functions [30,31]. The induction of IRF-7 in infection leads to rapid IRF-7 assembly and activation by phosphorylation [32]. Homo- or hetero-dimerization with IRF-3 results in nuclear entry and enhanced type I IFN expression, as well as the induction or upregulation of ISGs [29].

IRF-7 is also involved in other virus recognition pathways [33–35]. A family of sensor proteins called TLRs are able to recognize PAMPs and can then subsequently induce IRF-7 [36]. TLR7 and TLR9 are located in the endosomal membrane and signal via the adapter molecule Myeloid differentiation primary response gene 88 (Myd88) [36]. A

complex of different kinases assembles around the adapter which also recruits and phosphorylates IRF-7 [37]. This again leads to the induction of type I IFNs [37]. Another viral sensor known to recognize viral RNA is TLR3 [38]. This endosomal receptor signals downstream along the IKKε/TBK1 axis via the adapter molecule TIR-domain-containing adapter-inducing interferon-β (TRIF) and can also induce a type I IFN response [38].
A signaling cascade that has been shown to support IFN production as well, is the mTOR pathway which promotes the activation of IRF-7 [33]. So far it is unclear if this is mediated by viral entry [33].
By viral recognition the NF-κB pathway can be activated by Myd88/IRF-5 and TBK1/IKKε complexes [39]. It was shown that the IRF-7 promotor contains a Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) binding site and therefore might be directly regulated by NF-κB [40]. Overall the induction of NF-κB leads to a signaling cascade that plays a role in inflammatory responses as well as cell proliferation and apoptosis and therefore antiviral defense [36].

1.1.2. The Interferon system

A class of immunomodulatory cytokines that are able to induce an antiviral state in infected as well as uninfected cells by innate immunity is the interferon family [41]. Activated by different signaling pathways, these cytokines exhibit a broad range of functions with an impact on cellular differentiation, growth and the production of defense molecules [41,42]. So far, three different subgroups of IFNs have been characterized. The type I IFN family is composed so far of 13 different IFN-α genes and 1 IFN-β gene, which are structurally and functionally related [43]. Less well known members like IFN-δ in pigs and IFN-τ in ruminants are species specific or like IFN-κ in keratinocytes and IFN-ε in placenta, region or cell type related [44–46]. Type I IFNs bind to the IFNAR, which is expressed nearly all cells [47]. Therefore all cells of the organism are able to react to type I IFN stimulation [47].
IFN-γ is the only member of the type II IFN family, which displays antiviral function but no structural homologies to the type I IFN group [43]. The heterodimeric receptor that is bound by IFN-γ consists of the IFN-γ gamma receptor 1 (IFNGR1) and IFN-γ receptor 2 (IFNGR2) chains [43]. IFN-γ is mainly secreted by activated T cells as well as NK cells and plays an important role in macrophage activation and the induction of major histocompatibility complex (MHC) expression [48,49].
A group of cytokines known before as Interleukin-29 (IL-29, IFN-λ1), IL-28A (IFN-λ2) and IL-28B (IFN-λ3) has been assigned to the IFN-λ family or type III IFNs [50]. These bind to

the IFNLR1 and IL-10Rβ receptor which are expressed mainly by epithelial cells. Mucosal tissues are therefore a specific target for type III IFNs [50].

The signaling of Interferons is mediated by the binding of individual receptors. Nevertheless, similar or even same signaling pathways are activated via the phosphorylation of Stat proteins by IFN signaling. Therefore identical or overlapping ISG profiles are induced by different IFN types (Fig.1) [51]

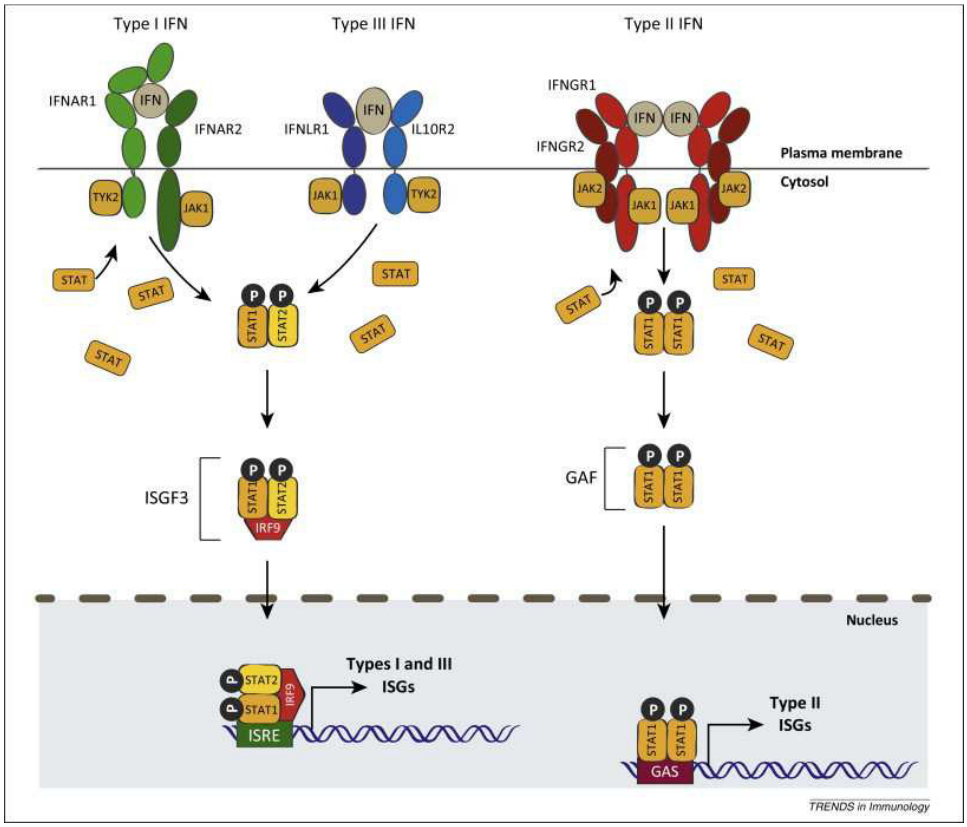

Fig.1: Jak/Stat pathway signaling by Interferons. Type I, II and III IFNs bind to different receptor complexes but signal collectively via the JAK/STAT pathway. Different Stats homo- or heterodimerize upon phosphorylation and induce gene transcription by binding of ISRE or GAS sequences in antiviral genes. This leads to differentiated and individual antiviral responses against various pathogens [51].

Activated Stat complexes bind to genes that contain ISRE or Gamma-IFN activated sites (GAS). This can be achieved by direct IFN signaling or by the induction of ISGs that bind to these sequences. Additionally, even though type I IFNs are bound to IFNAR1-IFNAR2 complexes and signal mainly by Stat1/2 heterodimers which bind to ISRE sequences, also Stat1 and Stat3 homodimers can be accumulated by IFN-α and IFN-β. This leads to binding of a different gene set with GAS sequences. Different IFN families are therefore able, by receptor preference and Stat complex activation, to induce distinct but also

overlapping transcription profiles which subsequently leads to broader immune responses [51,52].

1.1.3. Interferon regulatory factors (IRFs)

IRFs are a family of transcription factors that are induced by IFN signaling or direct PAMP recognition of the cell [53]. These factors are very diverse in their function but share a N-terminal DNA-binding domain (DBD) [26]. They induce a broad range of innate immune responses by different signaling pathways [54,55]. Some are constitutively expressed whereas others have to be induced and differences have been characterized for different cell types [32][55]. IRFs mediate activating or repressive functions which results in gene induction that is associated with cell growth, apoptosis or differentiation [55–58]. IRF-1 is a transcriptional activator that binds to cis elements of the IFN-β promotor and regulates the expression of various other inflammatory factors like MHC class I, IFN-α, OAS, protein kinase R (PKR), Caspase-1 and IL-15 genes [30][59]. IRF-2 provides a negative feedback loop for IRF-1 by transcriptional inhibition of IRF-1 induced genes [30]. This impacts cellular processes such as cell death or cell differentiation [30]. IRF-1 and IRF-2 are expressed in many different cell types [30][60].

In contrast, IRF-6 expression is cell type restricted. It can mainly be found in skin and is needed for keratinocyte differentiation. It therefore plays a role in skin and limb formation [55].

Another factor of the family, IRF-4, was identified originally in lymphoid cell populations [61]. IRF-4 influences many processes like B cell proliferation and T cell differentiation but also plays a role in PU.1 mediated macrophage signaling [61–63]. Similarly to IRF-4, IRF-8 is only expressed by immune cells and interacts with transcription factor PU.1 [55]. It is responsible for various differentiation and proliferation processes in the myeloid cell population [55][64]. IRF-8 also activates the NF-κB signaling pathway in a TLR9 dependent manner in DCs [65]. This NF-κB pathway is also initiated by IRF-5 signaling [66]. IRF-5 is e.g. expressed in B cells, DCs and monocytes and signals downstream of viral recognition by TLR7, TLR9 and TLR4 [67]. IRF-5 has also been shown to induce different and distinct subsets of IFN-α genes [68]. Interestingly, IRF-5 is not induced equally by viruses since it was activated by Newcastle disease virus (NDV) but not in Sendai virus (SeV) infection [68].

IRF-3, IRF-7 and IRF-9 execute different and distinct functions in the type I IFN induction pathway like described briefly above. Due to their important role in the IFN induction pathway, the function of IRF-3 and IRF-7 proteins will be addressed in more detail.

Virus mediated IFN induction by IRF-3 and IRF-7

IRF-3 and IRF-7 are important for effective and robust IFN responses [31][69]. IRF-3 was found to be merely responsible for the early IFN response, characterized by IFN-β and IFN-α4 production [70]. In contrast, IRF-7 induces a positive feedback loop and therefore leads to an amplification of the IFN signal [69,70]. Both factors activate different and distinct gene subsets and show overlapping but non redundant functions [26][69]. This sort of fine tuning for the IFN response is mediated by a regulation of IRF-7 on several levels [26]. IRF-3 is constitutively expressed in all tested cell types whereas IRF-7 has to be induced in most populations [26]. The exception are plasmacytoid DCs (pDCs), where basal expression of IRF-7 facilitates a fast type I IFN response [71]. Therefore genes that are only activated by IRF-7 but not IRF-3 can be considered as a delayed effector subset [69]. Different splice variants of IRF-7 also result in a more diverse protein subset [72]. However, mostly the 67 kDa mature IRF-7 protein can be found in differentiated cells and especially in lymphocytes [72]. In contrast, IRF-3 is only the size of 55 kDa [26]. Interestingly, both proteins show very different half-lives in the cell [69]. IRF-3 is very stable whereas IRF-7 contains an internal degradation signal which results in the low half-live of roughly an hour and a relative self-limiting response [69].
Another level of regulation is added by posttranslational modification [26]. Both proteins are phosphorylated on specific serine residues on their C-terminus [26]. This leads to a conformational change which results in the inactivation of intramolecular auto-inhibitory IRF associated domains (IAD) and also to a dimerization of these proteins [26]. Homo- or heterodimerization of IRF-3 and IRF-7 induce distinct subsets of IFN genes which show slight variances in their ISRE sequences [69]. IRF-7 has been shown to selectively induce IFN-α2, IFN-α5, IFN-α6 and IFN- α8, whereas IRF-3 activates mainly IFN-β and IFN-α4 expression [27]. IRF-3 also readily recruits Co-activators like cAMP responsive element-binding protein (CREB) which was not shown for IRF-7 [73]. This leads to signal amplification and fine tuning of antiviral responses [26][73].

The role of IRF-3 and IRF-7 in viral infections

The loss of IRF-3 and IRF-7 can result in impaired immune responses by the host [71]. It was shown that cells lacking IRF-3 or IRF-7 are more receptive to viral infections and that the Interferon production is decreased or abolished [54][71]. *In vivo* mouse models that lack IRF-7 have been associated with lower systemic IFN responses in blood [54][71]. Additionally, a much higher susceptibility of these animals to different virus infections has been shown [54][71]. These effects might be due to differentiation defects in monocytes

which depend on IRF-7 for differentiation [74]. Another factor might be the delayed or impaired IFN production by pDCs, which are considered to be the main source for type I IFN [71]. In most cases these animals succumb to infection rapidly, for instance in herpes simplex virus -1 (HSV-1), West Nile virus (WNV) and Encephalomyocarditis virus (EMCV) infection [54][71]. Mutations in the IRF-7 gene in humans has also been associated with severe Influenza in younger children [75].

1.2. Tick Borne Encephalitis Virus

Infections with viruses of the *Flaviviridae* such as dengue virus (DENV), WNV, Japanese encephalitis virus (JeV), Zika virus (ZIKV) and tick-borne encephalitis virus (TBEV) are known to cause severe illness in humans all over the world [76,77]. In the last decades some of these viruses have attracted attention as emerging or re-emerging pathogens providing a risk to public health [78–80].

1.2.1. Relevance of TBEV as emerging pathogen

TBEV is mainly distributed across Asia and Europe and considered to be the most widespread arthropod-borne disease in central Europe [81]. Infections with TBEV are recorded in nearly all European states, but travel related infections into some of these countries have been noted [82,83]. Many infections go unnoticed not only because of the often subclinical course, but also the low awareness, like e.g. in Bulgaria [84]. A case study of patients presenting with encephalitis of unknown origin revealed TBEV positive cases [84]. This is especially important, since TBEV is one of the emerging diseases with case numbers increasing since 1990 [85]. Approximately 10.000-12.000 severe clinical cases are recorded annually in 30 different countries [85]. But not only case numbers, also the affected area is constantly expanding. In Germany the number of risk areas increased from 97 in 2005 to 137 endemic areas in 2012 [85,86]. Therefore, the European Commission added TBE to the list of communicable diseases for epidemiological surveillance in 2012 [87].

1.2.2. Clinical manifestation

The onset of symptoms is registered 7-14 days post infection, but might be shorter in case of infected milk consumption [88,89]. TBEV can lead to very different clinical outcomes in the infected host, depending on subtype, infection route and immune status of the host

[reviewed 88]. It is estimated that approximately 70-95% of the infections are subclinical and therefore not registered [90]. Other patients present with unspecific symptoms like headaches, nausea, fatigue and fever [88]. Infection with European strains often leads to a biphasic syndrome. The second phase affects 20-30% of the patients and the onset can emerge years after the primary infection but is characterized by more severe symptoms with impacts on the central nervous system (CNS) [88]. Patients can suffer from severe muscular pain, numbness, headaches, hearing defects and spinal paralysis leading to hemiplegia [88]. These symptoms are mostly due to the encephalitis or meningitis triggered by the virus and can lead in around 46 % of the clinical cases to neurological long term sequelae [91]. Though, case fatality rates are comparably low [88]. In contrast, infection with Far Eastern subtypes present with a monophasic disease where patients suffer from a more severe impact on the CNS with a much higher mortality [88]. Effective vaccination based on formalin inactivated European strains Neudoerfl (FSME Immun, Baxter) and K23 (Encepur, GlaxoSmithKline GmbH & Co. KG) are available in Europe [92,93]. A vaccination campaign in Austria reduced the number of TBEV cases very effectively [94]. Vaccination is a good precautionary measure since there is still no specific treatment licensed for TBEV [94]. This limits therapeutic options to symptomatic treatment [88].

1.2.3. Classification

Tick borne transmitted viruses are summarized in a group that varies in phylogeny, geographical distribution and the type of disease that is induced [95,96]. The classical TBEV subgroup is then clearly separated from phylogenetic similar viruses like Langat virus (LGTV), Powassan virus (POWV) and Omsk hemorrhagic fever virus (OHFV) [97,98]. All these viruses subtypes show a common structure, which is shared by all *Flaviviridae* [99].

TBEV subtypes

Overall three main groups have been defined: the Far-Eastern, the Siberian and the European subtypes [100]. Strains from different subgroups differ considerably regarding their characteristics like transmission vector or pathogenicity [88]. Neuroinvasiveness is much lower in Siberian subtypes compared to Far-Eastern strains like Sofijn [100,101]. Therefore case fatality rates range substantially between groups. Whereas Far-Eastern

strains show a fatal outcome in 20-40% of the cases, Siberian and European subtypes are less virulent with 7-8% and >2% fatality rates respectively [96].

Langat virus as a model for TBEV

The TBEV complex also includes virus strains that cluster close to these three subgroups but are considered as independent species [95][102][103]. One example is naturally attenuated LGTV [104]. This strain was originally isolated in Malaysia and shares 82-88% homology to the strains of the TBEV group [105]. Therefore strain TP21 was originally considered for different vaccination approaches [106,107]. Good neutralizing antibody responses especially against the conserved E Protein have made LGTV a promising candidate. Since some volunteers that received the active virus or the inactivated vaccine developed meningoencephalitis vaccination with TP21, this approach was aborted [88][108]. The LGTV strain TP21 still exhibits neurovirulent properties in rodents, especially in immune deficient animals such as severe combined immunodeficiency $(SCID)^{-/-}$ and $IFNAR^{-/-}$ mice and can therefore be used to mimic neurotropic TBEV infections [20][88][109–111].
Coupled with its relative harmlessness to humans under natural conditions, LGTV is used as experimental model for TBEV which can be handled under biosafety level 2 (BSL2) conditions [88]. In contrast, experimental work with regular TBEV strains is restricted to laboratory conditions at least on a BSL3 level [112].

Virus structure

Viruses of the TBEV group are positive charged, ssRNA viruses with an approximate size of 11 kilo base pairs [88][109]. A single open reading frame (ORF) encodes for a polyprotein that is cleaved into 3 structural (C, E, M) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [88]. The outer shell structure of the virion consists of the capsid protein (C), additionally to the TBEV E Protein, that forms the envelope [88]. The E Protein is a glycoprotein that interacts with receptors, mediates cell entry and can protect the virions from nucleases [88]. Nevertheless, the envelope protein can also elicit immune responses by the host [88]. The membrane protein (M) is first enclosed in the virion and only cleaved from a preM to the mature M protein when the virus particle leaves the host cell [88]. The TBEV virion, with a diameter of 50 nm also contains nucleic acids that encode for proteins with different functionalities [88]. NS5 is the largest and most conserved non-structural protein with a methyltransferase activity as well as an RNA polymerase function. It associates with the serinprotease NS3 to a virus

replication complex [88]. NS1 and NS2A fixate this complex to the membrane, whereas NS2B is considered to be a Co-factor for NS3. NS4 then helps to orientate the polyprotein in the host cell membrane [reviewed 23].

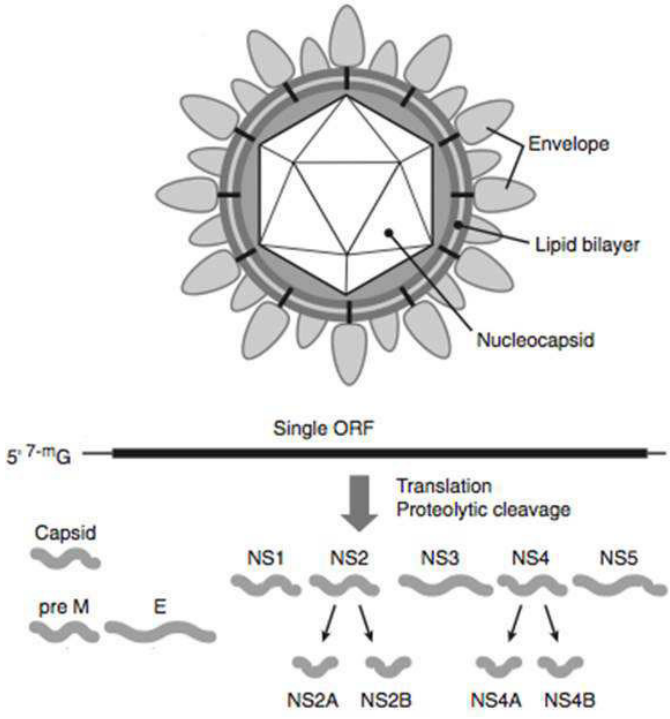

Fig.2: Basic structure of a Flavivirus. Langat virus shares the basal structure of all *Flaviviridae.* The Nucleocapsid contains non-structural proteins 1-5 (NS1-5) which are surrounded by the Capsid (C). The particle is organized as icosahedral spheric structure. Envelope (E) and Membrane (M) proteins are anchored in the lipid bilayer and detectable by the immune system. Figure was modified by L. Zegenhagen [113].

1.2.4. Infection with viruses of the TBEV group

Viruses of the TBEV group are mostly transmitted by tick bite of *Ixodes ricinus*, but few cases are reported where the virus was incorporated by infected goat milk. Ticks favor a broad spectrum of vectors like rodents, deer, sheep, cows, moose but also humans [84][114]. In consent with a tick bite as transmission route, migrating monocytes and macrophages of the skin are possible target cells. These cells are known to be vehicles for systemic virus spread which is a common mechanism shown for other viruses [115–117]. TBEV and LGTV can infect a broad range of immune cells like B cells, neutrophils and DCs, additionally to monocytes and macrophages [20][118]. However, the cell type that is most susceptible to LGTV infection are neurons [20][119,120]. LGTV is a neurotropic and neuroinvasive virus but the mechanism by which the virus enters the CNS and therefore gains access to the highly susceptible neuronal tissue is still unclear. Since

immune cells can be infected, it is possible that the virus enters the brain via the ´Trojan horse mechanism´ [20]. On the other hand, a brief viremia that was shown for other arboviruses might be sufficient for the virus to enter the CNS [121]. Since neurons are so susceptible to LGTV infection, the travelling of the virus via peripheral nerves like sensory olfactory neurons can also not be excluded [20]. Other flaviviruses like WNV have been shown to use several routes to enter the protected site of the CNS [122–124]. The virus induced for instance a high production of inflammatory cytokines that leads to a breakdown of the blood brain barrier (BBB) [122]. In contrast, in LGTV infection it was shown recently, that the virus can be detected in the brain already before the BBB is breached [20]. In this context, defects in recognition in LGTV like the absence of TLR7 have been linked with enhanced viral replication and increased CNS infection in mice *in vivo* [125]. Mutations in the TLR3 gene have been associated with more severe TBEV disease in humans [126].

Once the virus reached the brain, different neuropathological effects can be observed, including perivascular cuffing and necrotic or degenerating cells in different brain regions [127]. This also includes infiltration of immune cells into the CNS, independent of the viral strain that was used, since it was observed for LGTV and Far Eastern strain Oshima [20] [127]. The effect in mice and humans is at least partly mediated by cytokines and cytokine receptors [128,129]. Inflammatory or anti-inflammatory cytokines have been shown to affect immune responses in the brain [127]. Cytokines like chemokine (C-C motif) ligand-4 (CCL-4), CCL-5, chemokine (C-X-C motif) ligand (CXCL-9) and CXCL-10 are upregulated in LGTV infection [127]. Tumor necrosis factor-alpha (TNF-α) and C-C chemokine receptor type 5 (CCr5) were even employable to distinguish dying animals from survivors [127,128].

Infiltrating cells differ in their potential role upon infection [128]. T cells have a protective function in TBEV infection whereas increased infiltration of neutrophils was associated with enhanced apoptosis in the CNS [128]. Neurons seem to be the key player in this situation [20][126][130]. Whereas Vero cells and Neurons are both susceptible to LGTV clone 636, only Neuro-2a cells showed a clear upregulation of cell death related genes and apoptosis [131]. In this context it is interesting to note, that TBEV replicates especially in neuronal dendrites. Abnormal swelling of neurites, membrane reorganization to elliptical structures and condensation of neuronal cytoplasma has been determined upon infection and this might also cause enhanced cell death [119]. This explains to some extent neurological symptoms and sequelae observed in patients [88].

1.2.5. Interference of TBEV and LGTV with the Innate Immune system

Many flaviviruses are known to interact and interfere with the immune response to escape innate recognition. TBEV is no exception and multiple strategies have been uncovered about how TBEV is able to circumvent or delay IFN induction. By hiding in vesicles of the endoplasmatic reticulum (ER), TBEV strains like Neudoerfl, Hypr and Absetterov are able to hide early replication from cytosolic sensors and therefore allow the virus a head start [132]. However, spillover from the replication products can be detected later in the cytosol by RIG-I in an IPS-1 dependent manner and leads to the induction of a delayed type I IFN induction by IRF-3 [132,133]. Downstream signaling of induced and secreted IFN that is bound by IFNAR is then impaired by TBEV NS5 protein. NS5 of LGTV binds to prolidase, a cytosolic dipeptidase responsible for the localization and maturation of IFNAR1 to the cellular membrane [134]. This limitation of receptor availability leads to a reduced sensing of initially secreted type I IFN [134]. Studies also implemented a direct interaction of LGTV NS5 with IFNAR2 which leads to reduced pStat translocation to the nucleus [135]. Similarly, NS5 of Hypr was shown to locate to the host cell membrane in a complex with human host protein scribble (hScrib), which led to impaired Jak/Stat pathway activation [136]. As a counter measure, host factor tripartite motif 79 alpha (TRIM79α) has been shown to selectively degrade NS5 of TBEV [137].

Nevertheless, even diminished levels of IFN can effectively induce antiviral factors. Therefore TBEV also counteracts several ISGs. It was shown that TBEV impaired DC maturation by the inhibition of IRF-1 translocation to the nucleus as well as the interference with IL-12 production. This was not only observed for attenuated LGTV but also for the more virulent TBEV strain Sofjin [138]. Thus, TBEV interferes with all parts of the type I IFN induction pathway and by this diminishes antiviral responses by the host.

1.3. Aim of the Thesis

Emerging viruses associated to the genus *Flavivirus* have attracted a lot of attention in recent years [78–80]. Vaccination strategies, treatment options and prevention measures against arthropod vectors are often limited and therefore the interest in new therapeutic options is high [2][81]. In this context, a specific focus has been placed on innate immune responses. Viruses counteract very different parts of the initial defence, especially the induction and amplification of type I IFNs [132][134].

Here, the TBEV strain LGTV was used as a model to study parts of the type I IFN signalling pathway in more detail. TBEV is an emerging pathogen on the northern hemisphere and even though vaccination strategies have been successful, risk factors for complications in neuropathology and neuroinvasiveness are still mostly unknown [78]. It was shown, that the loss of IFN-β is not critical for the survival of LGTV infection in mice [20]. However, the absence of a type I IFN response in IFNAR knock outs led to an onset of severe neurological symptoms similar to complications in humans, such as encephalitis and meningitis [20].

Since the loss of different signalling components led to such diverse disease outcomes *in vivo*, the function of further key molecules that are involved in this pathway was of interest. Therefore, early mediators of type I IFN induction and factors involved in signal amplification were analysed in a mouse model. The aim was to characterize the impact of deficits in the IFN pathway on type I IFN induction in the periphery to identify possible protective factors.

Since LGTV is a neurotropic virus, a specific focus of this work was also laid on infection mediated effects on the CNS in the absence of key factors of the IFN response. Very few information are available about the role of IPS-1, IRF-3 and IRF-7 in the brain, especially associated with TBEV or LGTV infection. Alterations in local immune responses in the absence of these factors were addressed and the type I IFN response of specific brain resident cells upon infection was characterized in more detail.

2. Results

2.1. Type I IFN signalling in Langat virus infection

The induction and maintenance of type I IFNs can be mediated by different signalling pathways [20][37]. This depends on the cellular location and the specific PRR that senses the virus [22][37]. Associated to the activated receptor the signal is transferred to specific adaptor molecules and further regulatory factors, which will initiate and enhance type I IFN signalling [26][37].

2.1.1. Significance of TLRs and RLRs in LGTV infection

Especially TLRs and RLRs have been shown to play an important role in the recognition of ssRNA viruses [37][139]. TLR7, TLR9 and TLR3 are bound to the endosomal membrane and transmit their signal via the adaptor molecule Myd88, or, in case of TLR3, TRIF [37].To test if TLRs or RLRs are important for the survival of LGTV infection, mice deficient for Myd88 and TRIF were infected and the survival analysed. Additionally the role of the RLRs RIG-I and MDA-5 were tested, which signal via the adapter molecule IPS-1 [25]. Genetically modified C57BL/6 mice deficient for Myd88, TRIF and IPS-1 were systemically infected by intraperitoneal LGTV injection (Fig.3).

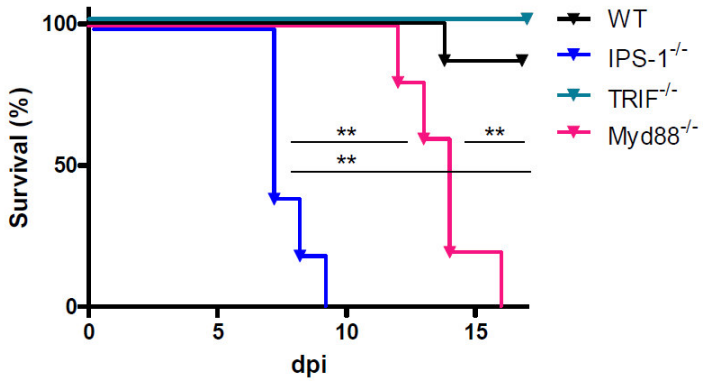

Fig.3: Role of RLR and TLR adaptor molecules Myd88, TRIF and IPS-1 in LGTV infection. Mice (n =5) deficient for IPS-1, Myd88 or TRIF and WT were infected intra peritoneally with $1x10^4$ FFU LGTV and monitored for survival. Statistical analysis was performed with Mantel Cox test, *** $p<0.0001$.

Most WT animals survive LGTV infection without any clinical symptoms. In contrast, mice defective for IPS-1 succumbed rapidly to infection after 7-9 days post infection (dpi) and

showed weight loss, hunchback, eye infections, fur ruffling and lethargy. In the absence of Myd88 all mice died within 12-17 dpi showing the same symptoms like IPS-1 animals. Like WT mice, TRIF$^{-/-}$ mice survive the infection with minor to no disease symptoms. This indicates that RIG-I and MDA-5 are important in the early phase, whereas the protective role of Myd88 dependent TLR7 and TLR9 is needed later during systemic LGTV infection. In addition, TRIF and therefore TLR3 signalling are dispensable for survival.

To determine if TRIF, Myd88 and IPS-1 have potentially synergistic effects, Myd88$^{-/-}$IPS-1$^{-/-}$, Myd88$^{-/-}$TRIF$^{-/-}$ and Myd88$^{-/-}$IPS-1$^{-/-}$TRIF$^{-/-}$ mice were infected systemically by intraperitoneal LGTV infection and monitored for survival.

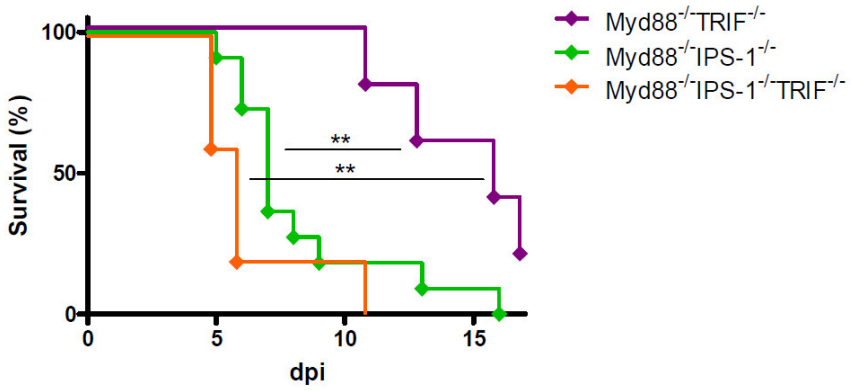

Fig.4: Synergistic effects of TLR and RLR adaptor molecules in LGTV infection. Myd88$^{-/-}$IPS-1$^{-/-}$ (n=11), Myd88$^{-/-}$TRIF$^{-/-}$ (n=5) and Myd88$^{-/-}$IPS-1$^{-/-}$TRIF$^{-/-}$ (n=5) mice were infected i.p. with 1x10^4 FFU LGTV. Survival was monitored and statistical analysis was performed with Mantel Cox test, *** p<0.0001.

Myd88$^{-/-}$TRIF$^{-/-}$ mice died with a similar kinetic like Myd88$^{-/-}$ animals which underlines the negligible role of TRIF for LGTV infection in mice. Myd88$^{-/-}$IPS-1$^{-/-}$ and Myd88$^{-/-}$IPS-1$^{-/-}$ TRIF$^{-/-}$ mice showed a comparable kinetic in the survival curve like IPS-1$^{-/-}$ mice, suggesting that IPS-1 is important to control initial LGTV infection. Taken together, this indicates a distinct time line for the activation of RIG-I and TLR pathways upon LGTV infection.

2.1.2. Implications of distinct IRFs on the survival of LGTV infections

IRFs are the main regulatory proteins in the type I IFN response and are known to be crucial for the survival of many different viral infections [31][71]. IRF-3 is responsible for early IFN induction, whereas IRF-7 has to be induced in most cell types and mediates signal amplification of the type I IFN response [69]. To elucidate distinct roles of different

IRFs in LGTV infection knock out animals were inoculated intraperitoneally with the virus. The survival analysis of IRF-3, IRF-7 and IRF-5 deficient animals is shown below (Fig.5)

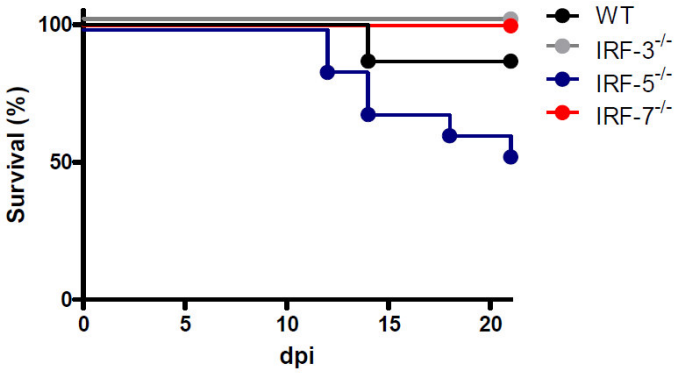

Fig.5: The influence of IRFs on the survival of LGTV infection. Survival analysis of WT (n=15), IRF-3$^{-/-}$ (n=15), IRF-5$^{-/-}$ (n=13) and IRF-7$^{-/-}$ (n=10) mice infected i.p. with 1x10^4 FFU LGTV. Data represent at least two independent experiments. Animals were monitored 21 days for survival. Statistical analysis was performed with Mantel Cox test.

All IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice survive systemic infections with LGTV, whereas approximately 40% of the IRF-5$^{-/-}$ animals succumb to infection, with symptoms such as hunchback, lethargy and weight loss. This shows that IRF-5 plays a protective role in LGTV infection, whereas IRF-3 and IRF-7 are not essential for overall survival. However, mice were monitored for disease symptoms and weight loss and differences between WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ animals can be detected during the course of the infection (Fig.6).

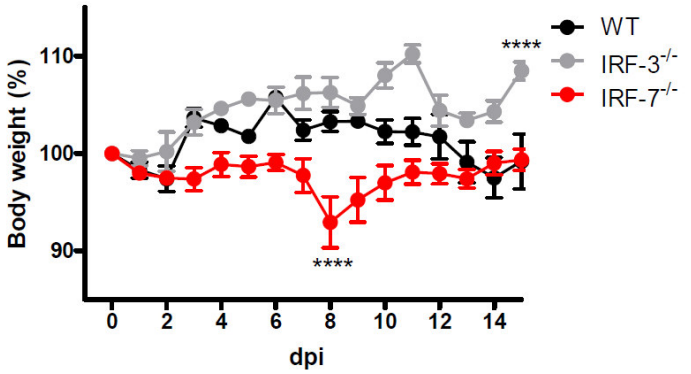

Fig.6: Weight loss of IRF deficient animals infected with LGTV. WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice were infected as before. Weight loss was determined daily until 15 dpi, calculated to the weight of the uninfected animal and represented in (%). Statistical analysis was performed by 1way ANOVA, *** p<0.0001. Statistical error is depicted as standard error of mean (SEM).

IRF-7$^{-/-}$ animals show a significant weight loss around 7-8 dpi in contrast to WT and IRF-3$^{-/-}$ mice. Interestingly, none of these mice show any other disease symptoms, like lethargy,

hunchback or fur ruffling. This data indicates that even though animals present as overall healthy, infection by LGTV affects IRF-7 deficient mice.

2.1.3. Type I IFN induction by LGTV in the absence of IRFs

The loss of type I IFN signaling components often impacts systemic responses to viral infections. IFNAR$^{-/-}$ animals for instance show higher IFN levels in serum of WNV infected animals [31].
Differences in systemic type I IFN responses in the absence of these IRFs have been noted in various infections [31][71]. In some cases the loss of IRF-3 and IRF-7 has no impact on serum levels at all, whereas decreased IFN serum levels have been noted in viral infections [29][31][54][71]. We therefore analysed serum of infected animals for the systemic type I IFN response upon LGTV infection (Fig.7).

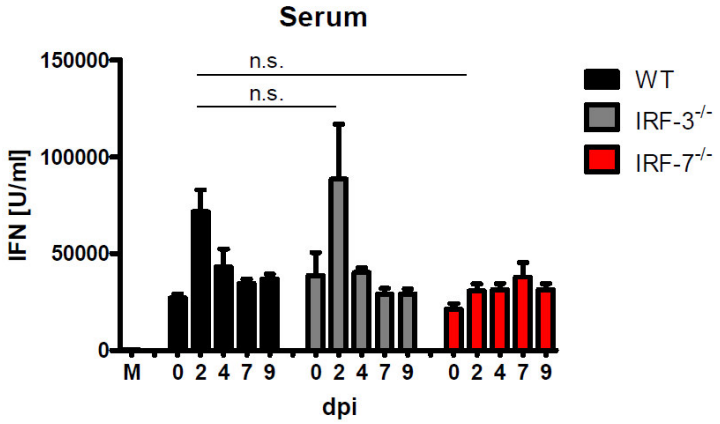

Fig.7: Loss of IRF-7 results in reduced systemic type I IFN responses. Blood was isolated from WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice (n=5) 0, 2, 4, 7 and 9 dpi. Inactivated serum was analysed for type I IFN by a luciferase based bioassay. Type I IFN was calculated based on a standard curve and is depicted as Units IFN per ml. Statistical analysis was performed by 1way ANOVA. Statistical error is shown as standard error of mean (SEM).

WT and IRF-3$^{-/-}$ animals show a systemic type I IFN response on 2 dpi. In contrast, the loss of IRF-7 abolishes type I IFN production in serum upon infection. These data are comparable with that shown in WNV infections, which also belongs to the flavivirus family [54].

2.1.4. Local type I IFN production in the periphery despite defects in IRFs

Type I IFN is often produced locally. To detect organ and region specific type I IFN responses, a luciferase-based IFN-reporter mouse strain (IFN-β$^{+/\Delta luc}$) was used. The

luciferase gene is expressed under the control of the IFN-β promotor. The heterogeneity of the gene locus ensures the production of IFN-β upon infection and allows simultaneous quantification of luciferin conversion by the equally induced luciferase [140]. Animals were infected with LGTV and IFN-β expression was quantified in several organs and results for spleen (Fig.8A), thymus (Fig.8B) and lung (Fig.8C) are shown below.

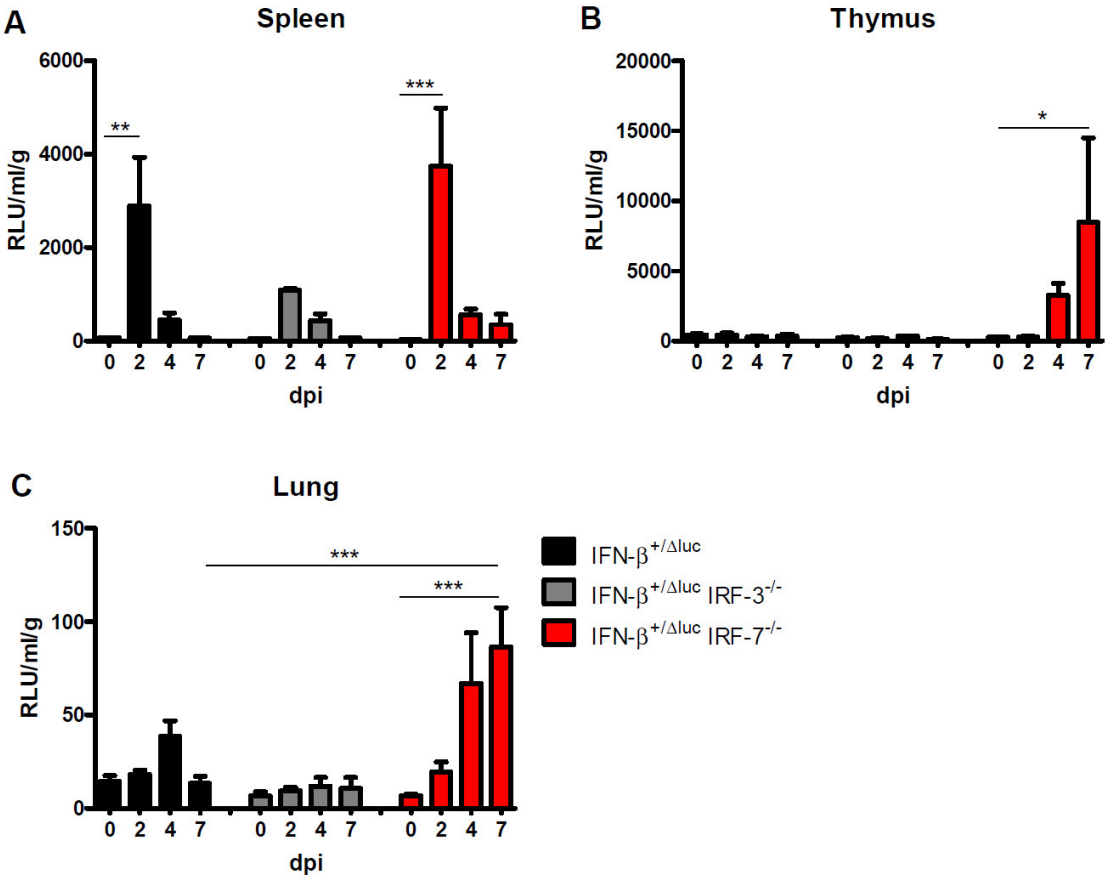

Fig.8: Local IFN-β induction in LGTV infected reporter mice. IFN-$\beta^{+/\Delta Luc}$, IFN-$\beta^{+/\Delta Luc}$ IRF-$3^{-/-}$ and IFN-$\beta^{+/\Delta Luc}$ IRF-$7^{-/-}$ mice (n≥5) were infected i.p. as described and spleen (A), thymus (B) and lung (C) were isolated 0, 2, 4 and 7 dpi for quantification of luciferase expression. Luciferase level are shown as Relative light units (RLU) per ml and were normalized to 1 g tissue. Statistical analysis was performed by 1way ANOVA, *** p<0.0001. Statistical error is shown as standard error of mean (SEM).

Type I IFN is induced in spleen and lung but not in the thymus of WT IFN-$\beta^{+/\Delta luc}$ animals at 2 or 4 dpi. Even lower IFN level were detected in IFN-$\beta^{+/\Delta luc}$ IRF-$3^{-/-}$ animals. However, despite the absence of the positive feedback loop for IFN which is normally initiated by IRF-7, these animals were able to induce high local IFN-β expression levels. Spleens of IFN-$\beta^{+/\Delta luc}$ IRF-$7^{-/-}$ animals show comparable IFN-β level to IFN-$\beta^{+/\Delta luc}$. Interestingly, in thymus and lung the IFN-β response was increased to a later time point but significantly

higher than in IFN-β$^{+/\Delta luc}$ mice. Overall lung showed here the lowest induction of type I IFNs.

Even though type I IFN in serum is reduced in LGTV infection, type I IFN can be induced locally even in the absence of IRF-7. Secondary lymphoid organs like spleen behave like WT, whereas primary lymphoid tissues like the thymus only show an IFN response in the IRF-7 knock out. To determine the behaviour of other secondary lymphoid organs, the type I IFN response was evaluated in lymph nodes and nasal associated lymphoid tissues (Nalt) (Fig. 9).

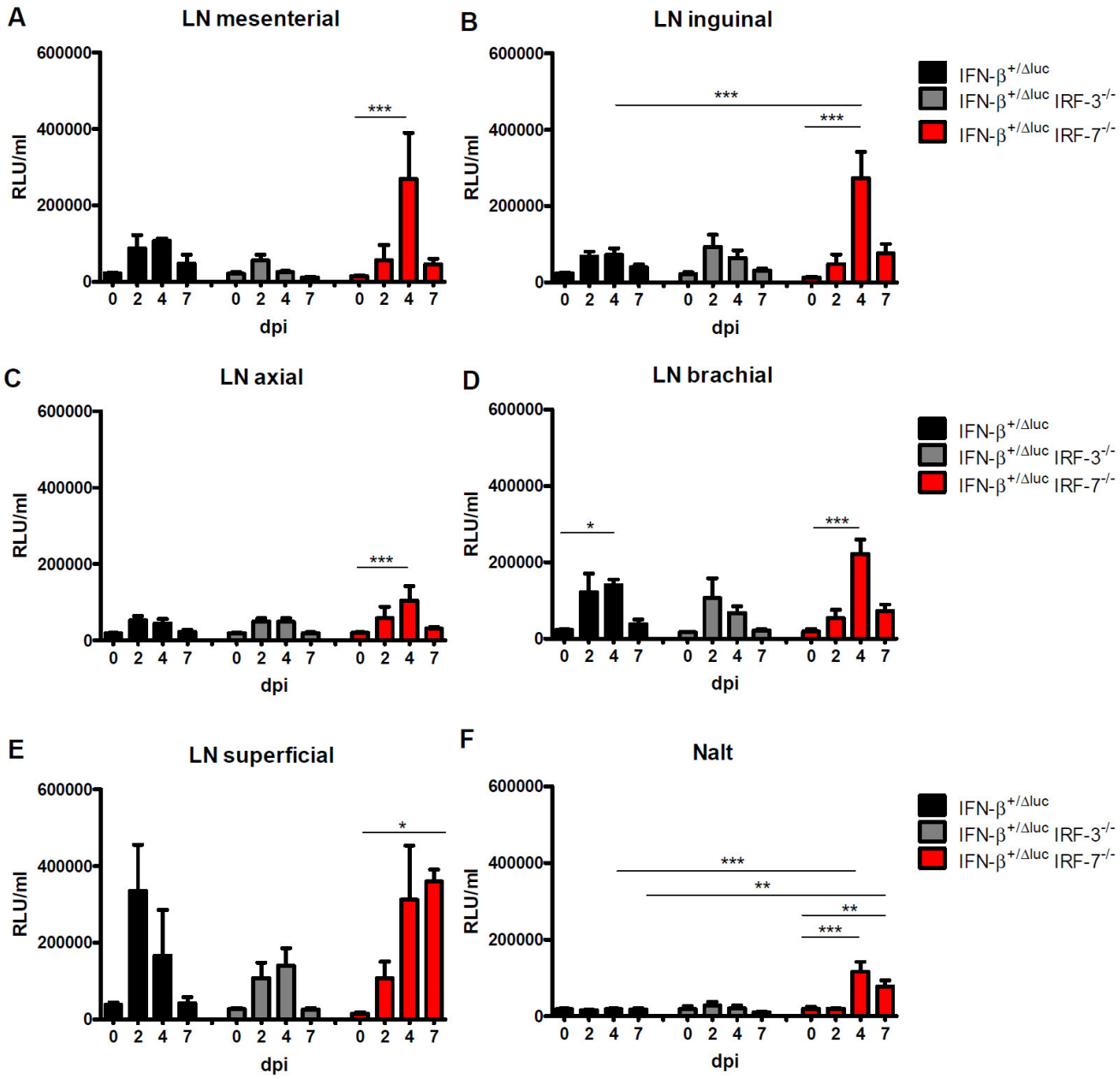

Fig.9: Induction of IFN-β promotor in secondary lymphoid tissues in the absence of IRFs. Different lymphoid tissues were isolated from intraperitoneally with LGTV infected IFN-β$^{+/\Delta Luc}$, IFN-β$^{+/\Delta Luc}$ IRF-3$^{-/-}$ and IFN-β$^{+/\Delta Luc}$ IRF-7$^{-/-}$ mice (n≥5) at 0, 2, 4 and 7 dpi. Luciferase expression of

mesenteric (A), inguinal (B), axial (C), brachial (D) and superficial (E) lymph nodes (LN) as well as nasal associated lymphoid tissue (Nalt) were quantified by bioassay and determined as RLU/ml. Statistical analysis was performed by 1way ANOVA, *** $p<0.0001$. Statistical error is shown as standard error of mean (SEM).

IFN-β was induced in lymph nodes of different origin at 2-4 dpi. The strength of the luciferase signal is comparable between mesenterial, inguinal, axial and brachial lymph nodes (A-D) in IFN-$\beta^{+/\Delta luc}$ mice. The signal was highest in the superficial lymph nodes (E) found in the environment of the salivary glands and lowest in lymphoid tissue of the snout and axial lymph nodes (C, F). This shows that intraperitoneally infected animals respond to the virus in a systemic manner. The response from IFN-$\beta^{+/\Delta luc}$ IRF-$3^{-/-}$ animals was slightly lower but else comparable to IFN-$\beta^{+/\Delta luc}$ mice.
IFN-β promotor coupled luciferase induction was again much higher in the absence of IRF-7, compared to IFN-$\beta^{+/\Delta luc}$ and IFN-$\beta^{+/\Delta luc}$ IRF-$3^{-/-}$ animals (A-F). The induction in IFN-$\beta^{+/\Delta luc}$ IRF-$7^{-/-}$ peaked around 4 dpi and decreased again to 7 dpi. As seen in superficial lymph nodes (E), the induction of type I IFN was realized earlier in infection compared to delayed induction of IFN-$\beta^{+/\Delta luc}$ IRF-$7^{-/-}$ tissues. This data shows that IFN-β expression is induced upon LGTV infection in an IRF-7 independent manner. This is in contrast to further observations during viral infection with other viruses. For this reason the significance of LGTV infection on peripheral organs was analysed in more detail.

2.2. IRF dependent impact of systemic LGTV infection on the periphery

Deficits in the type I IFN response have been associated with elevated virus replication in peripheral organs and therefore higher viral loads [20]. To validate if these changes can be seen in the LGTV infection model of IRF-$3^{-/-}$ and IRF-$7^{-/-}$ mice as well, different aspects of peripheral infection processes were dissected.

2.2.1. Morphological changes in lymphoid organs in the absence of IRFs

LGTV and TBEV can cause different macroscopic changes in peripheral organs upon infection. Light patches on the liver have been observed and can be associated with liver damage. Parts of the intestinal tract were affected. Icteric intestines, expanded guts probably due to intestinal congestion and hemorrhaging in the small intestine and gall bladder are visible in highly infected animals (observations of L.Zegenhagen, S.Schreier). Therefore, WT, IRF-$3^{-/-}$ and IRF-$7^{-/-}$ animals were analysed thoroughly for tissue

abnormalities. Here, isolated lymph nodes and spleens from different time points during the infection are shown (Fig. 10 and Fig. 11).

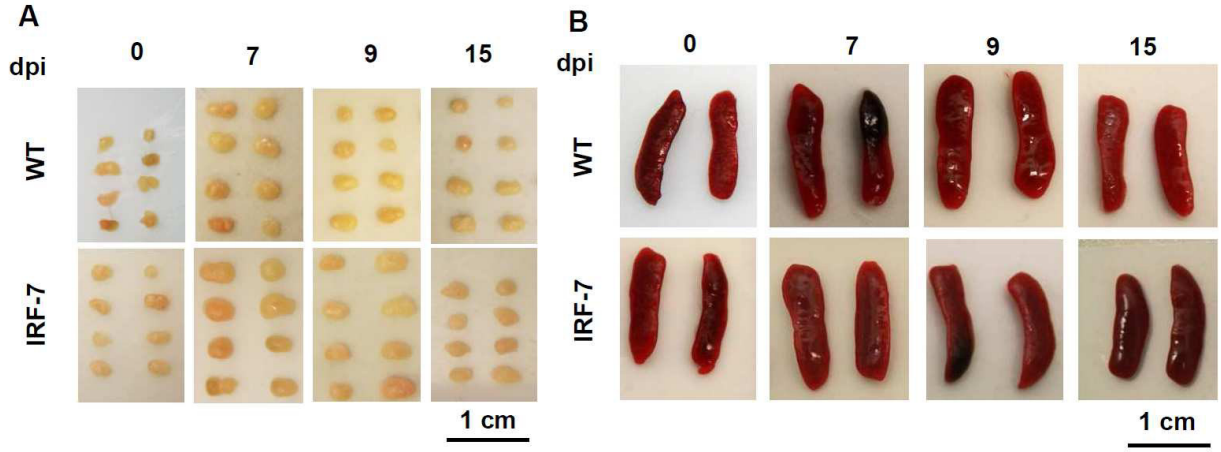

Fig.10: Size gain of spleens and lymph nodes in LGTV infected animals. Inguinal (top) and brachial (bottom) lymph nodes (A) as well as spleen (B) from uninfected and systemically with 1x 10^4 FFU LGTV infected WT and IRF-$7^{-/-}$ animals were isolated at 0, 7, 9 and 15 dpi (A). For each time point and genotype two representative mice are shown. Scale bar is indicated (1cm).

Lymph node swelling and elevated spleen size were observed in acutely infected animals as a phenomenon that was independent of the genotype. These alterations can occur in acute immune responses and are caused by leukocytes proliferation and cell recruitment [141,142].

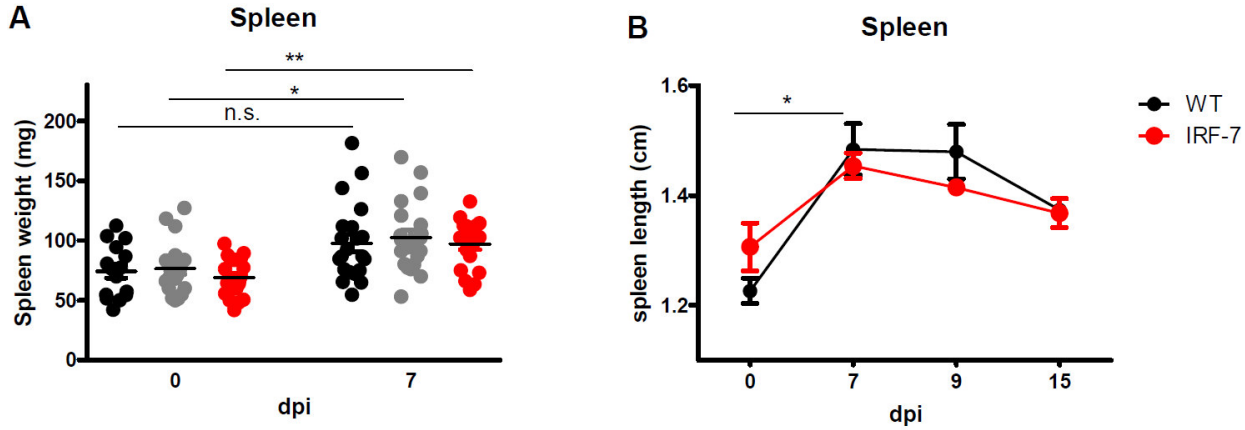

Fig.11: IRF dependent alterations in spleen dimensions upon LGTV infection. Spleens of i.p. with LGTV infected WT, IRF-$3^{-/-}$ and IRF-$7^{-/-}$ animals were analysed for alterations in weight (n ≥15) (A) and in WT and IRF-$7^{-/-}$ spleens for differences in length (n ≥3) (B). Statistical analysis was performed with 1way ANOVA, *** $p<0.0001$ (A) and t test (B), *** $p<0.0001$. Statistical error is shown as standard error of mean (SEM).

An increase in spleen weight (A) and length (B) caused by systemic LGTV infection was seen in WT animals, 7 dpi. A similar and significantly elevated spleen weight was also noted for IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice upon infection. The size of the spleen, represented here as spleen length, was enhanced in WT and IRF-7 animals in a similar manner and decreased in the later course of infection, as also seen in Fig.10 and 11.
Animals show a clear splenomegaly and lymph node hyperplasia along with enhanced type I IFN responses (Fig.8 and 9) which indicates an activation of the immune system upon systemic LGTV infection that affects different peripheral organs.

Macroscopic changes often result in the alteration of microscopic structures. To determine if the structural integrity of spleen was still intact and if there are changes in the number of immune cells harboured by the organ, spleens were histologically analysed.
Spleens function as filter organs and the parenchyma consists of white and red pulp. Whereas red pulp takes mainly part in the blood filtration, lymphocytes of the white pulp are able to recognize potential pathogen associated antigens that may be present by peripheral blood cells [143]. The white pulp appears as follicular structures which are additionally enclosed by a marginal zone and further surrounded by red pulp. These structures contain different lymphocytes populations. Upon infection this framework can disperse due to massive cell proliferation and cell migration. Therefore spleen microscopy was evaluated for WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$. Spleens of highly infected IFNAR$^{-/-}$ animals were used as controls (Fig.12).

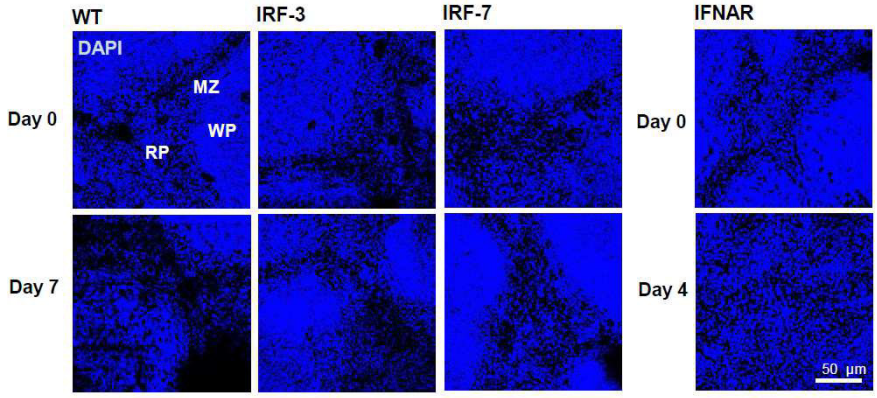

Fig.12: Impact of LGTV infection on spleen integrity. WT, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and IFNAR$^{-/-}$ spleens of uninfected and systemically LGTV infected animals were analysed for LGTV induced structural alterations. Organs were fixed and sectioned by cryotome into 30 µm slices. Cell nuclei were stained with DAPI. A representative white pulp (WP), marginal zone (MZ) and red pulp (RP) are indicated. Pictures were taken at 20x magnification and a scale bar is indicated.

The framework of WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ spleens was intact independent of LGTV infection. In contrast, spleen integrity of infected IFNAR$^{-/-}$ animals was dissolved and white pulp, marginal zones and red pulp areas were no longer distinguishable.

Even though no differences in the overall organ structure were observable, in the absence of IRFs, variations in the location of different cell populations might occur. White pulp follicles contain B cells, T cells as well as macrophages, DCs and reticular cells [143]. These cells are arranged in specific zones and changes can be detected in differentiated immune responses [143–145]. Therefore we analysed splenic follicles for the presence and dissemination of immune cells. Cell nuclei were additionally stained to allow structural orientation (Fig. 13).

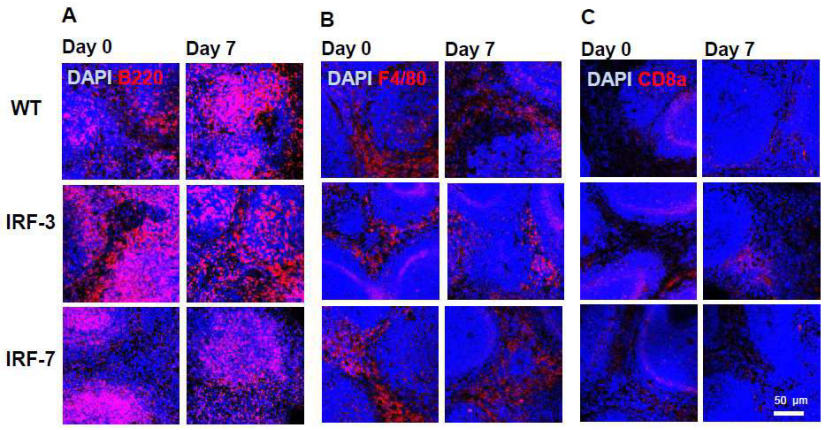

Fig.13: Histological analysis of spleen integrity upon LGTV infection. Spleens were isolated from systemically LGTV infected animals (n=3). After fixation, organs were embedded and 30 µm thick spleen slides obtained by cryotome sectioning. Spleen sections were stained for surface antigens B220 (A, B cells and DCs), F4/80 (B, macrophages) and CD8a (C, cytotoxic T cells and DCs). Nuclei were stained with DAPI. Pictures were taken at 20x magnification and scale bar is indicated (50 µm).

High numbers of B220 positive cells (A) could be detected, especially inside of the white pulp follicles of all analysed spleens. F4/80^{+} macrophages (B) were mainly observed in the marginal zone of lymphoid follicles. The same was true for CD8^{+} cytotoxic T cells (C). Overall, no differences in microscopic spleen structure and immune cell localisation were determined in infected and uninfected animals in the absence of IRFs.

2.2.2. The impact of IRFs on immune cell populations in infected spleens

Associated to the splenomegaly, alterations can be expected in the number of spleen cells and the composition of different leukocyte populations. Therefore myeloid splenocytes that may play a role in innate immune defences were analysed by flow cytometry (Fig.14).

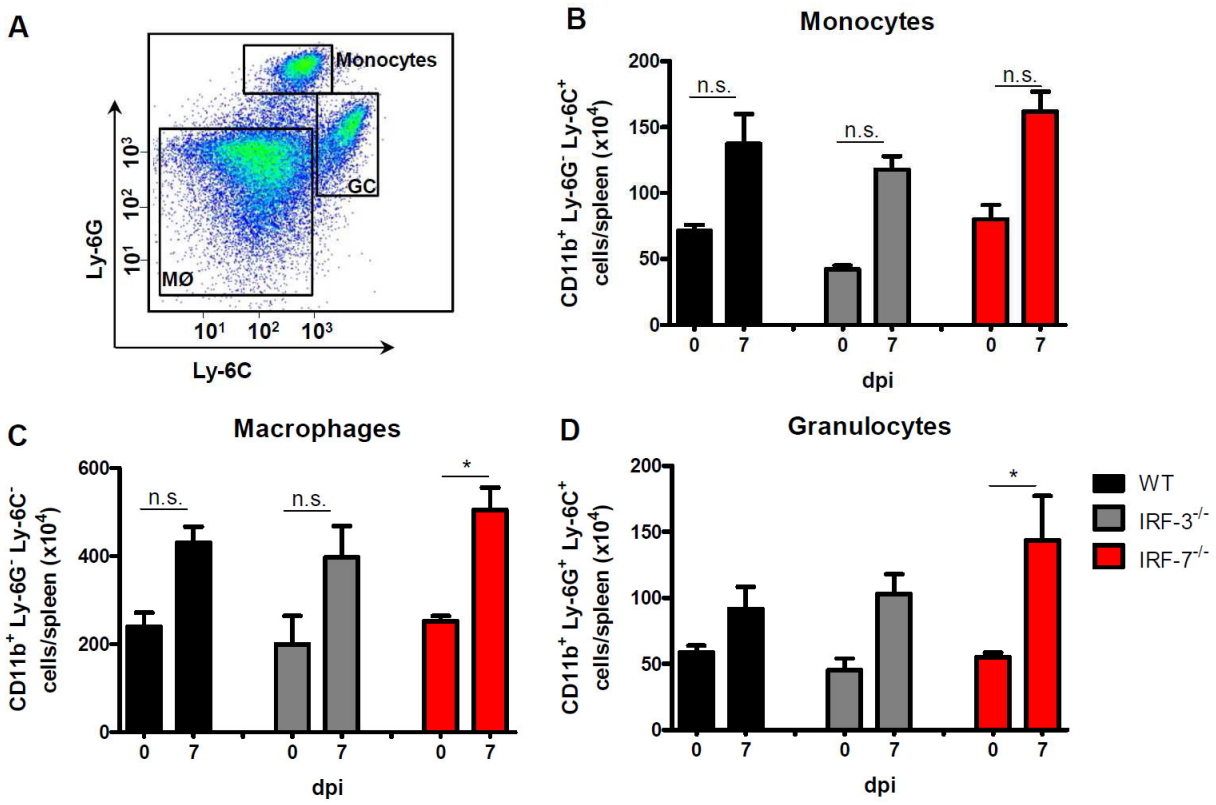

Fig.14: Differences in myeloid splenocyte populations in the absence of IRFs in LGTV infection. Splenocytes were obtained from spleens of systemically with 1x 10^4 FFU LGTV infected (7dpi) and uninfected WT, IRF-$3^{-/-}$ and IRF-$7^{-/-}$ animals (n=5). Splenocytes were characterized according to their surface marker expression as monocytes, macrophages (MØ) and granulocytes (GC) (A). Cells were identified as $CD11b^+Ly6G^-Ly\text{-}6C^+$ monocytes (B), $CD11b^+Ly6G^-Ly\text{-}6C^-$ macrophages (C) and $CD11b^+Ly6G^+Ly\text{-}6C^+$ granulocytes (D). Splenocytes were counted and cell populations were calculated to the total number of spleen cells. Statistical analysis was performed by 1way ANOVA, *** $p<0.0001$. The statistical error is shown as standard error of mean (SEM).

The overall number of monocytes, macrophages and granulocytes increases due to LGTV infection in all genotypes (B-D). Significantly higher numbers of granulocytes and macrophages but nor monocytes were found in spleens of infected IRF-$7^{-/-}$ animals (C, D). These cells are described as effector cells that can be mobilized from spleen in high numbers to inflammatory sites [146,147]. This indicates elevated inflammatory processes upon LGTV infection in IRF-$7^{-/-}$ animals compared to WT and IRF-$3^{-/-}$ mice. Of note, IRF-7 has been described as differentiation factor for monocytes *in vitro* [74]. No reduced macrophage numbers have been detected in our mouse model. However, a reduction in phagocytic activity was not analysed in these cells and can therefore not been excluded [74].

Other phagocytic cells and cells related to the adaptive immune response also play a role in inflammatory processes [9][148]. Therefore the frequency of NK cells, T cells and B cells in spleens upon LGTV infection was analysed (Fig.15).

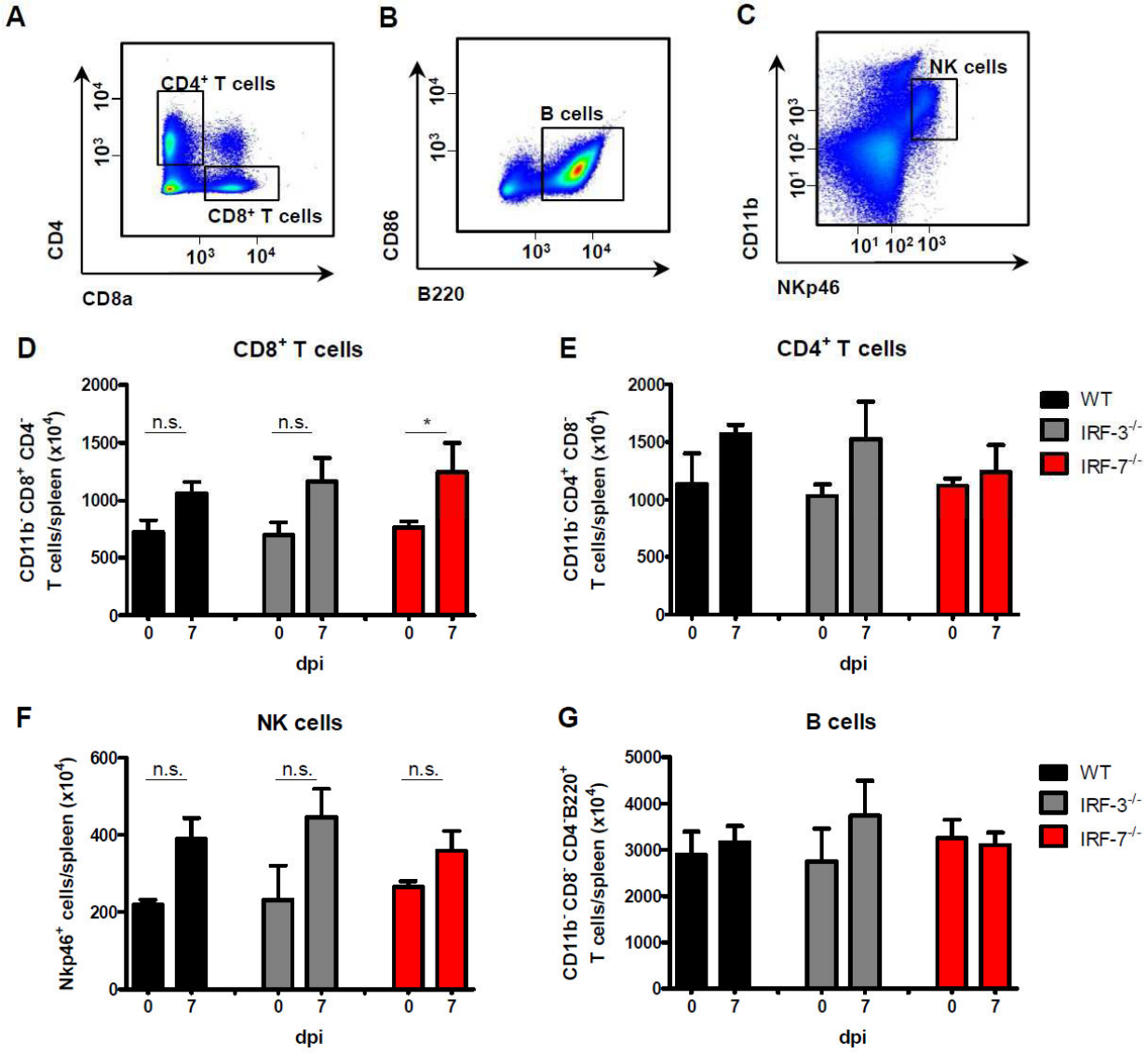

Fig.15: Alterations in splenocyte frequencies upon LGTV infection. Spleens were isolated from systemically LGTV infected (7dpi) and uninfected WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice (n=5). Splenocytes were analysed according to surface antigens (A, B, C) and quantified as CD11b$^-$CD4$^-$ CD8a$^+$ T cells (D), CD11b$^-$CD8$^-$CD4$^+$ T cells (E), Nkp45$^+$ cells (F) and CD11b$^-$CD4$^-$CD8a$^-$B220$^+$ B cells (G). Cells were calculated to the total number of splenocytes. Statistical analysis was done with 1way ANOVA, *** p<0.0001 and statistical error is shown as standard error of mean (SEM).

Similar to monocytes, macrophages and granulocytes, numbers of T cells and NK cells expand due to infection with LGTV (D-F). Still, this is only significant for CD8$^+$ T cells in IRF-7$^{-/-}$ animals (D). In contrast, B cell numbers are nearly unaffected by LGTV infection

(G). In summary, it can be concluded that the elevated spleen weight is attributable to an expansion of different immune cell populations upon infection. Still, the exact role of individual cell subsets in LGTV infection is not yet defined even though it has been described that different hematopoietic subsets can be infected by LGTV [20].
To determine if cells of the spleen are overall more susceptible to LGTV infection in the absence of IRF-3 and IRF-7, viral loads were determined (Fig.16).

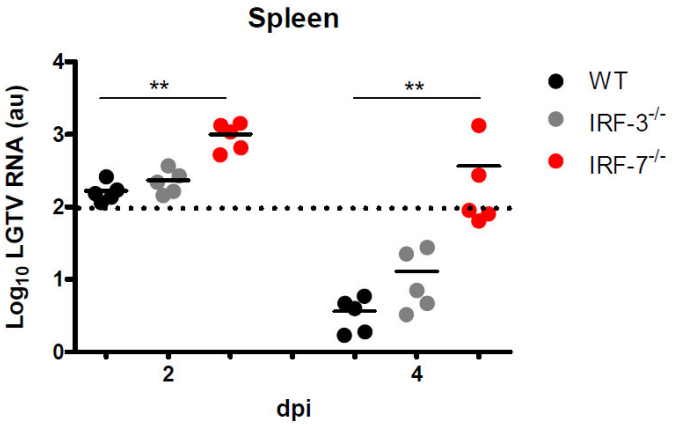

Fig.16: IRF-7 exhibits a protective function in spleen. Spleens were isolated from uninfected and systemically with LGTV infected WT, IRF-$3^{-/-}$ and IRF-$7^{-/-}$ animals (n=5). Viral RNA was determined by qRT-PCR detecting a specific NS3 sequence. CP values were normalized to the housekeeping gene β-Actin and relative expression calculated to uninfected WT samples. Statistical results were obtained by Mann-Whitney test, *** p<0.0001.

Viral loads were enhanced in WT samples of spleen at 2 dpi but decrease in the course of infection. Viral titers in spleens of IRF-$3^{-/-}$ animals behave similarly. The virus load in IRF-$7^{-/-}$ splenocytes was already significantly higher at 2 dpi compared to WT, indicating that these cells were more susceptible to LGTV infection. In contrast to WT and IRF-$3^{-/-}$, viral titers do not decrease in IRF-$7^{-/-}$ spleens in the course of infection. Therefore restriction of viral replication and viral clearance were impaired in the absence of IRF-7.

2.2.3. IRFs protect hematopoietic cells from LGTV infection

Macrophages, B cells and DCs can be infected by LGTV [20] and splenocytes show elevated viral level (Fig.16). Therefore the susceptibility of specific hematopoietic cells to LGTV in the absence of IRF-3 and IRF-7 was of interest. Since the specific focus lies here on defects of the type I IFN system, the aim was to elucidate the ability of IFN producing cells (IPCs) to resist LGTV infection. Therefore cells were isolated and differentiated from the bone marrow of WT, IRF-$3^{-/-}$ and IRF-$7^{-/-}$ animals and differentiated toward different

IPC populations. Bone marrow derived macrophages (BMDMs) and different DC populations namely pDCs and mDCs were used for infection experiments (Fig.17).

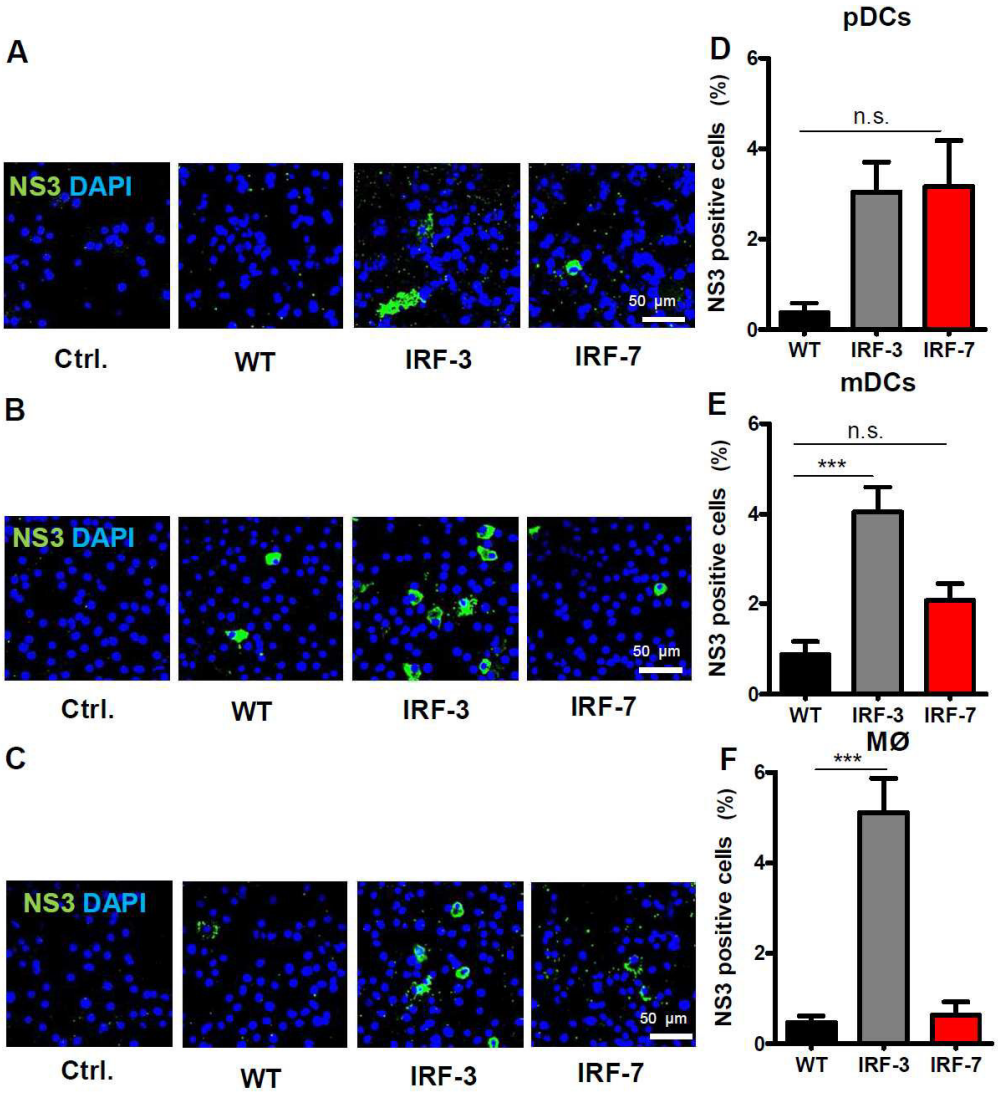

Fig.17: IRF-3 protects BMDCs from LGTV infection *in vitro*. Bone marrow derived cells were differentiated to pDCs, n≥5 (A, D), mDCs, n≥10 (B,E) and macrophages, n≥8 (C,E). Cells were infected with LGTV, multiplicity of infection (MOI) 0.5. Cells were fixed and stained with DAPI and viral anti-NS3 antibody 72 hpi. Displayed are uninfected (Ctrl.) and infected cells. Pictures were taken at 40x magnification and a scale bar is indicated (50 µm). Infected cells were counted and calculated to DAPI positive cells as (%). Statistical analysis was done with 1way ANOVA, *** p<0.0001. The statistical error is shown as standard error of mean (SEM).

WT cells showed only a very low infection rate from 0.5 to 1%, which indicates a low susceptibility of these cells to LGTV infection. In contrast, IRF-3$^{-/-}$ cells showed a relatively high infection rate *in vitro*. IRF-3$^{-/-}$ mDCs and macrophages are significantly higher infected than corresponding WT and IRF-7$^{-/-}$ cells. However, IRF-7$^{-/-}$ pDCs represent infection rates similar to IRF-3$^{-/-}$ pDCs at 72 hpi.

This elevated susceptibility of bone marrow derived IRF-3$^{-/-}$ cells clearly differs from *in vivo* data in spleen, where viral loads in IRF-7$^{-/-}$ splenocytes (Fig. 16) were increased compared to WT and IRF-3$^{-/-}$. Therefore local antiviral factors might play a role in the *in vivo* protection of IRF-3$^{-/-}$ animals.

2.2.4. The role of IRFs in the type I IFN production of hematopoietic cells during LGTV infection

Especially pDCs but to some extent also macrophages have been shown to elicit type I IFN responses [71][149]. Since very few of the BMDCs were infected, these cells might be protected by the induction of IFN. To evaluate the capacity of these populations to induce protective immune responses in the absence of IRF-3 and IRF-7, we tested these cells for their ability to secrete type I IFN (Fig.18).

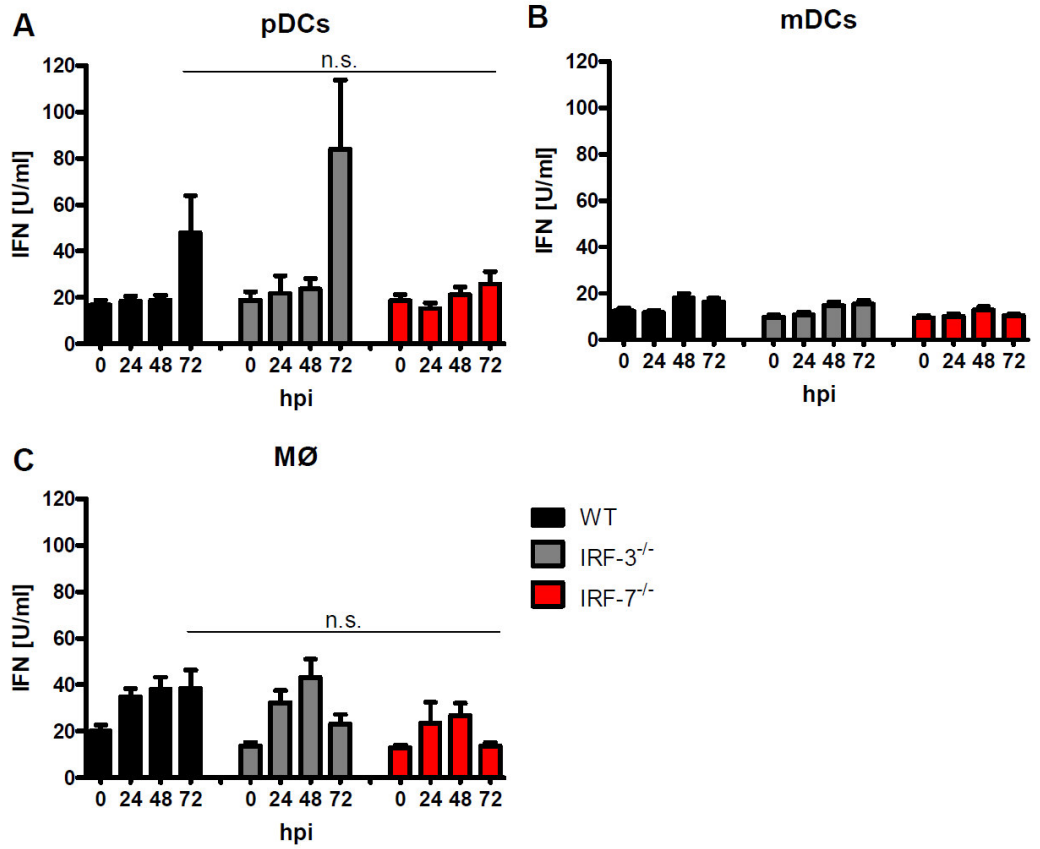

Fig.18: Impaired Type I IFN responses in BMDCs in the absence of IRF-7. Differentiated WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ BMDCs were infected with LGTV (MOI 0.5) and tested in a bioassay for their ability to induce type I IFN. Shown are pDCs, n≥11 (A), mDCs, n≥16 (B) and macrophages, n≥7 (C). Supernatants of these cells were analysed 24, 48 and 72 hpi and uninfected cells used as controls. Type I IFN was quantified based on a standard curve. Shown are at least two independent experiments. Statistical analysis were performed with 1way ANOVA, *** $p<0.0001$. Statistical error is shown as standard error of mean (SEM).

Overall, pDCs showed the highest induction of type I IFNs which is according to literature [71][150]. However, due to the constitutive expression of IRF-7, WT pDCs should be able to induce high amounts of type IFN much faster than other cells (A). Here, WT macrophages induce type I IFN earlier although in a lower and steadier manner (C). MDCs in contrast, induce relatively low amounts of type I IFN compared to macrophages and pDCs (B).
In the absence of IRF-3, type I IFN can be induced comparable to the WT situation. However, the loss of IRF-7 leads to abolished IFN responses in pDCs and decreased type I IFN levels in macrophages and mDCs. This is again according to literature, since high type I IFN production is known to be crucially dependent on IRF-7 [71]. Nevertheless, this data does not explain IFN-β expression that was seen in lymphoid organs (Fig.8 and 9) in earlier experiments. In contrast published data to WNV infection reflects the situation represented by type I IFN levels that have been detected in serum of IRF-$7^{-/-}$ animals (Fig.7) [54].

2.3. Specific functions of IRFs in neurotropic LGTV infection

LGTV is a virus with neuroinvasive and neurovirulent properties [20]. Even though the initial infection often occurs in the periphery, the potential damaging effects by LGTV are mainly due to the infection of the CNS [151]. To date it is unknown how Langat virus enters the brain. Infected hematopoietic cells from spleen (Fig.16) might act as “Trojan horses” or transient viremia might play a role in CNS invasion. Once the virus enters the brain, several local and protective immune responses can be induced by brain resident or infiltrating immune cells [151]. In this context, the local impact of IRF deficiency on distinct brain resident cells and the overall immune response was characterized in the CNS.

2.3.1. The influence of distinct IRFs on LGTV distribution in the CNS

Low and transient viremia is a phenomenon known from other arboviral infections and might promote viral dissemination of the virus in the periphery and invasion to the CNS [121]. For this reason viral loads were determined in serum of IRF deficient animals upon peripheral LGTV infection (Fig.19).

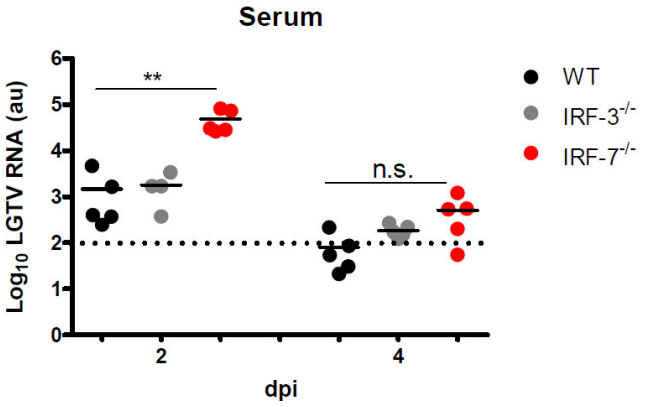

Fig.19: The loss of IRF-7 leads to transient viremia in LGTV infection. Blood was isolated from systemically infected WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice. Serum was tested for the presence of viral NS3 by qRT-PCR at 2 and 4 dpi. CP values were normalized to β-Actin and the relative expression was calculated based on uninfected WT samples. Results are displayed as arbitrary units and the dotted line represents signal intensity of uninfected controls. Statistical results were obtained by Mann-Whitney test, *** p<0.0001.

Viral levels in serum of WT animals decreased slightly in the course of infection, similarly to LGTV quantities in serum of IRF-3$^{-/-}$ and IRF-7$^{-/-}$ animals. Significantly elevated levels of LGTV NS3 were observed in the serum of IRF-7$^{-/-}$ animals at 2 dpi which decreased to 4 dpi. This indicates a transient viremia in IRF-7$^{-/-}$ animals.

A viremia can lead to enhanced dissemination of the virus into peripheral organs but also into the CNS. Since LGTV is a neurotropic virus differences in the neuropathology of IRF-7$^{-/-}$ animals was of specific interest and will be addressed in more detail.

Due to the location, different regions of the CNS show varying access to the immune system and provide the pathogens with several access options. The olfactory bulb represents the smaller anterior part of the CNS whereas the cerebrum is a comparably huge region with multiple functions such as cognitive control, processing of sensory stimuli and storage of information. The cerebellum handles all motor functions and lies on the posterior axis of the brain. Below, the brain stem is located, which is accountable for vegetative functions such as breathing and descends into the spinal cord. Due to variations in their accessibility for the virus via peripheral neurons, breaches of the BBB or cerebrospinal fluid barrier (CSFB) or the “Trojan Horse” mechanism all regions were tested separately for viral loads (Fig.18). To characterize the local impact of IRF-3 and IRF-7 on LGTV infection in the brain, viral loads were determined in different brain regions (Fig.20).

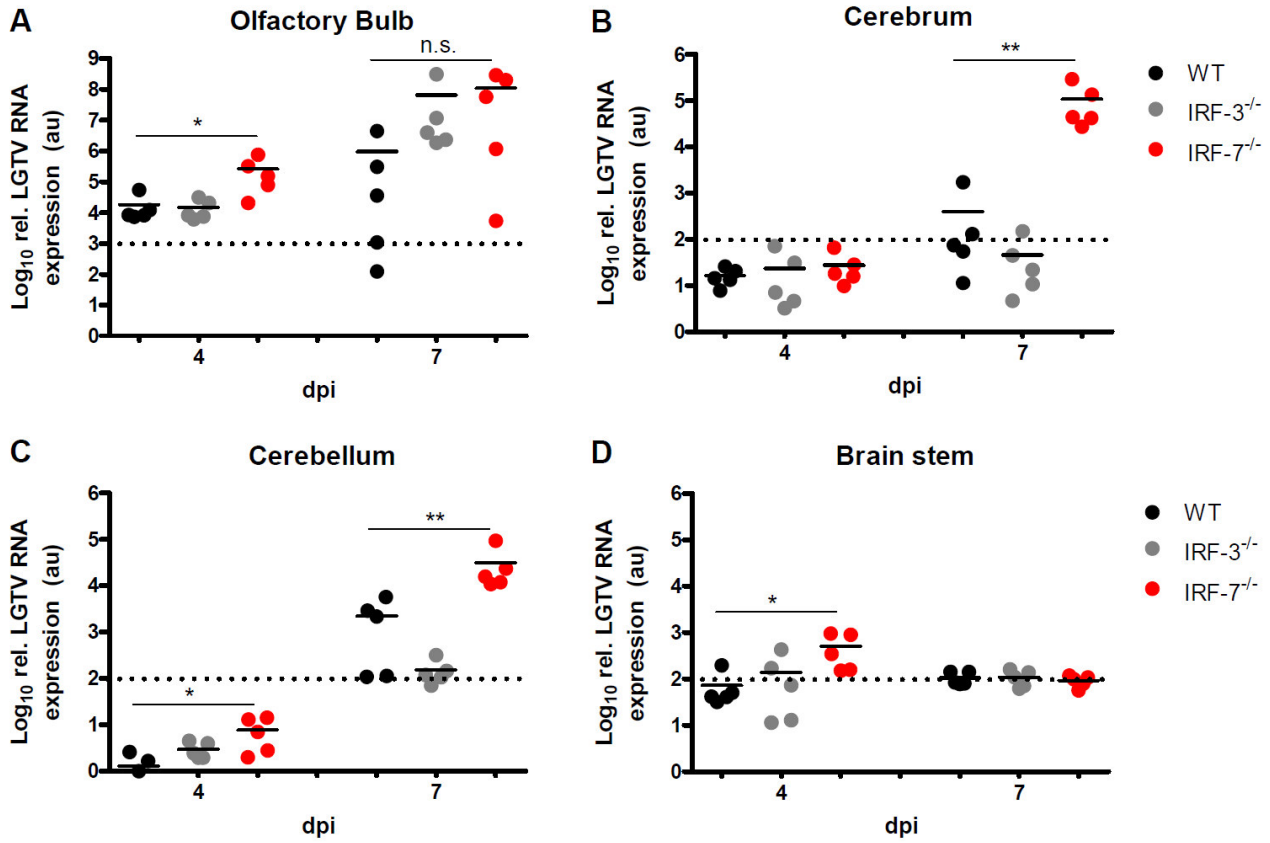

Fig.20: IRF-7 suppresses LGTV infection in the CNS. WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice were infected systemically with LGTV with 1x 10^4 FFU. Olfactory bulb (A), cerebrum (B), cerebellum (C) and brain stem (D) were analysed by q RT-PCR and relative expression of NS3 RNA was normalized to β-Actin and calculated to uninfected WT samples (dotted line). Results are indicated as arbitrary units (au). Statistical analysis was done by Mann-Whitney test, *** p<0.0001.

LGTV was detectable in all brain parts of WT animals and the signal was highest in the olfactory bulb (A) and lowest in brain stem (D). Viral amounts increased in the course of infection except for brain stem where viral signal remained very low (A-D). Enhanced viral replication was also seen in the olfactory bulb, cerebrum, cerebellum and brain stem of IRF-3$^{-/-}$ and IRF-7$^{-/-}$ brains from 4 dpi to 7 dpi. Whereas NS3 LGTV signals of IRF-3$^{-/-}$ mice were comparable to WT, the loss of IRF-7 led to higher viral signals in all brain parts. This suggests that IRF-7 acts as a restriction factor for viral replication in the brain.

Not only different brain regions but also different structures of the CNS can be targeted by different viruses. This is due to local differences in the cellular composition of the brain as well as differentially regulated regional immune responses [152]. To elucidate if specific brain areas are particularly affected by the loss of IFN regulating factors such as IRF-3 and IRF-7, histological stained sections of the brain were screened for the presence of viral E-protein (Fig.21).

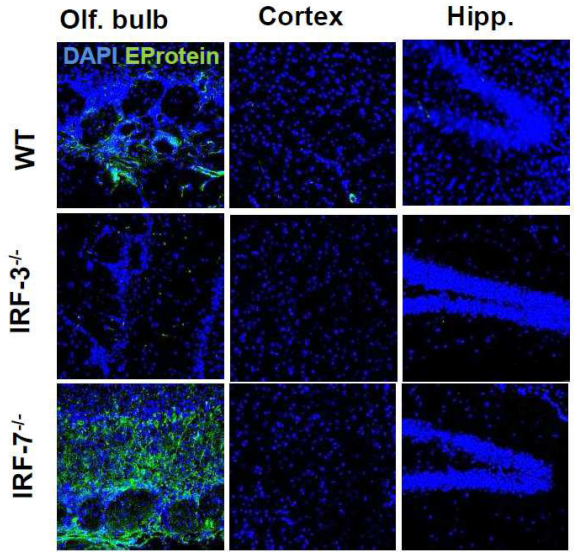

Fig.21: LGTV is disseminated equally in specific brain structures in the absence of type I IFN signaling components. Brain of uninfected or i.p. with LGTV infected WT, IRF-3$^{-/-}$and IRF-7$^{-/-}$ and mice (n≥3) were isolated 7dpi and stained for virus (E-Protein) and cell nuclei (DAPI). Shown are olfactory bulb (Olf. Bulb), cortex and hippocampus (Hipp.) and pictures were taken at 20x magnification and scale bar is indicated (200 µm). Representative pictures for at least three independent experiments.

The virus was mainly detected in the olfactory bulb region of WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ animals. Affected by LGTV was especially the glomerular cell layer which receives direct input from external sensory neurons. In the mitral cell layer and the granule cell layers of the olfactory system which are sequentially located, nearly no virus signal can be seen. Independent of the genotype, no reliable signals were detected in other representative brain regions such as cortex and hippocampus. Other specific brain structures for instance striatum, thalamus, midbrain, cerebellum and brain stem were checked but no virus signal was detected (data not shown). The RT-PCR represents therefore the more sensitive method for virus detection in this model.

The olfactory bulb of WT animals shows a much lower viral signal than IRF-7$^{-/-}$ mice. Nearly no signals were detected in IRF-3$^{-/-}$ brains.

IRF-7 was identified as a restriction factor of the type I IFN system that limits viral replication especially in the olfactory bulb region.

2.3.2. Immune cell related alterations in infection due to defective type I IFN responses

High viral replication in the CNS is often correlated with enhanced local inflammation. The associated production of pro-inflammatory cytokines then leads to the increased

recruitment of immune cells from the periphery. Cells such as CD8 T cells, macrophages and neutrophilic granulocytes invade the CNS upon infection [148][153,154]. These cells play a crucial role in the clearance of the infection but also in the induction of harmful neuropathology [154]. Therefore alterations in the invasion of immune cells into the CNS, in combination with a disturbed type I IFN response, were analyzed (Fig.21).

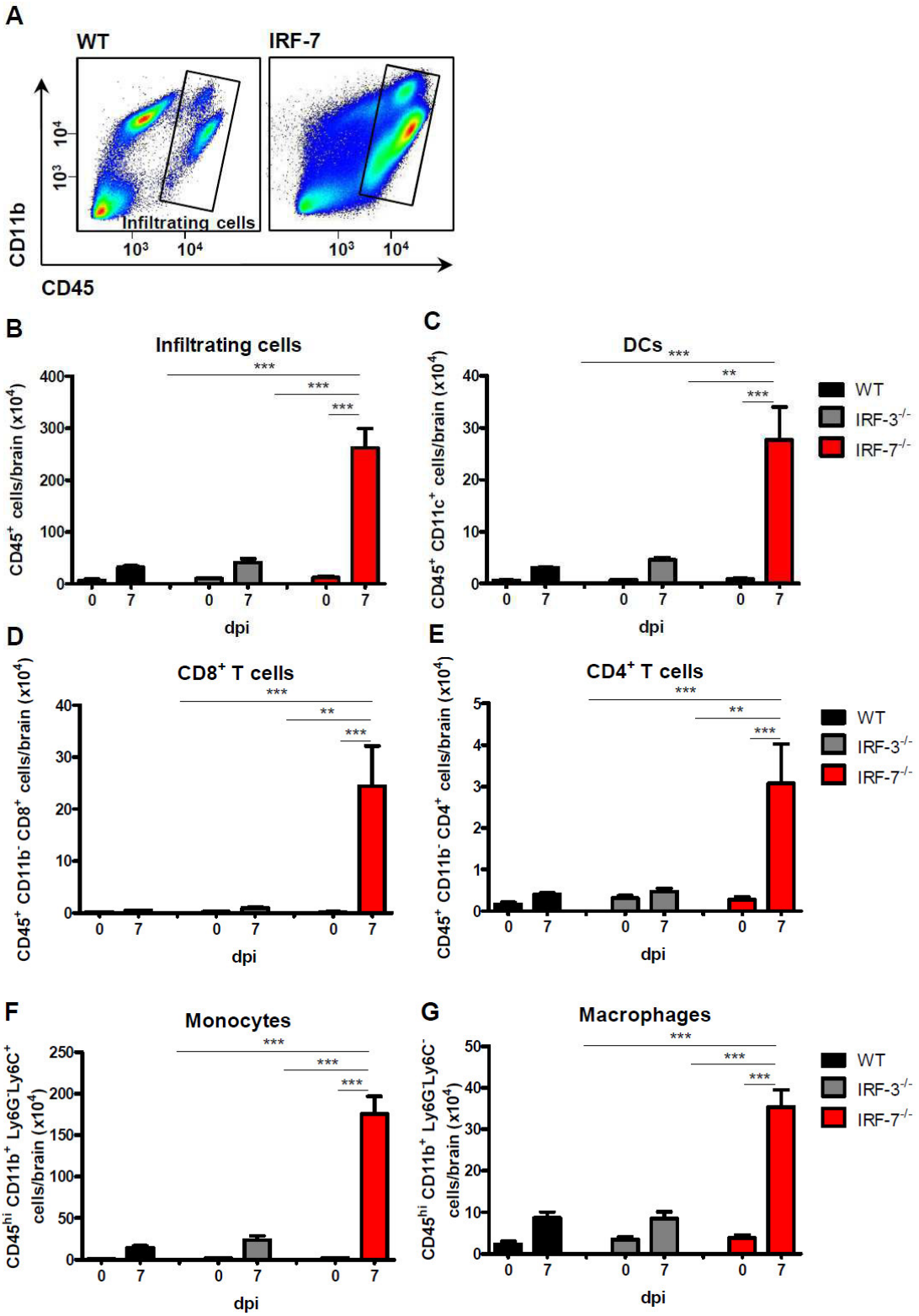

Fig.22: Impact of type I IFN deficiencies on immune cell infiltration into the CNS. Cells were isolated from brains of uninfected and systemically LGTV infected (7dpi) WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ (n=5) mice by percoll gradient and cell numbers were determined. Cell populations were charcterized by surface marker expression as CD45^{hi} infiltrating cells (A, B). Cells were further distinguished into CD45^{hi}CD11c^{+} DCs (C), CD45^{hi}CD11b^{-}CD4^{-}CD8^{+} T cells (D), CD45^{hi}CD11b^{-}CD8^{-} CD4^{+} T cells (E), CD45^{hi}CD11b^{+}Ly-6C^{-}Ly-6G^{+} monocytes (F) and CD45^{hi}CD11b^{+}Ly-6C^{-}Ly-6G^{-} macrophages (G) and calculated to total cells per brain. Statistical analysis was performed by 1way ANOVA, *** $p<0.0001$. Standard error of mean is depicted (SEM).

Only low number of immune cells can be found in the CNS of uninfected animals of the different genotypes. However, peripheral virus infection with LGTV leads to the infiltration of high numbers of different leukocytes into the CNS (A, B). Whereas in WT and IRF-3$^{-/-}$ only moderately enhanced cell counts were observed, IRF-7 shows a massive infiltration of leukocytes into the brain of infected animals 7dpi. Significantly elevated numbers of DCs (C), T cells (D, E) as well as monocytes (F) and macrophages (G) have been detected in the CNS of infected IRF-7$^{-/-}$ animals. This infiltration indicates a massive inflammatory impact on the brain of LGTVinfected IRF-7$^{-/-}$ animals.

Microglia are counterparts of monocytes and macrophages in the CNS [155]. These sentinels exhibit a surveillance function, phagocyte cell debris in the CNS, produce growth factor for cell survival or attract macrophages from the periphery to the CNS [155]. The activation of these cells by inflammatory processes in the CNS often results in a phenotype switch associated with a changed expression pattern of surface molecules [155,156]. Therefore microglia were analyzed for their frequency and activation status (Fig.23).

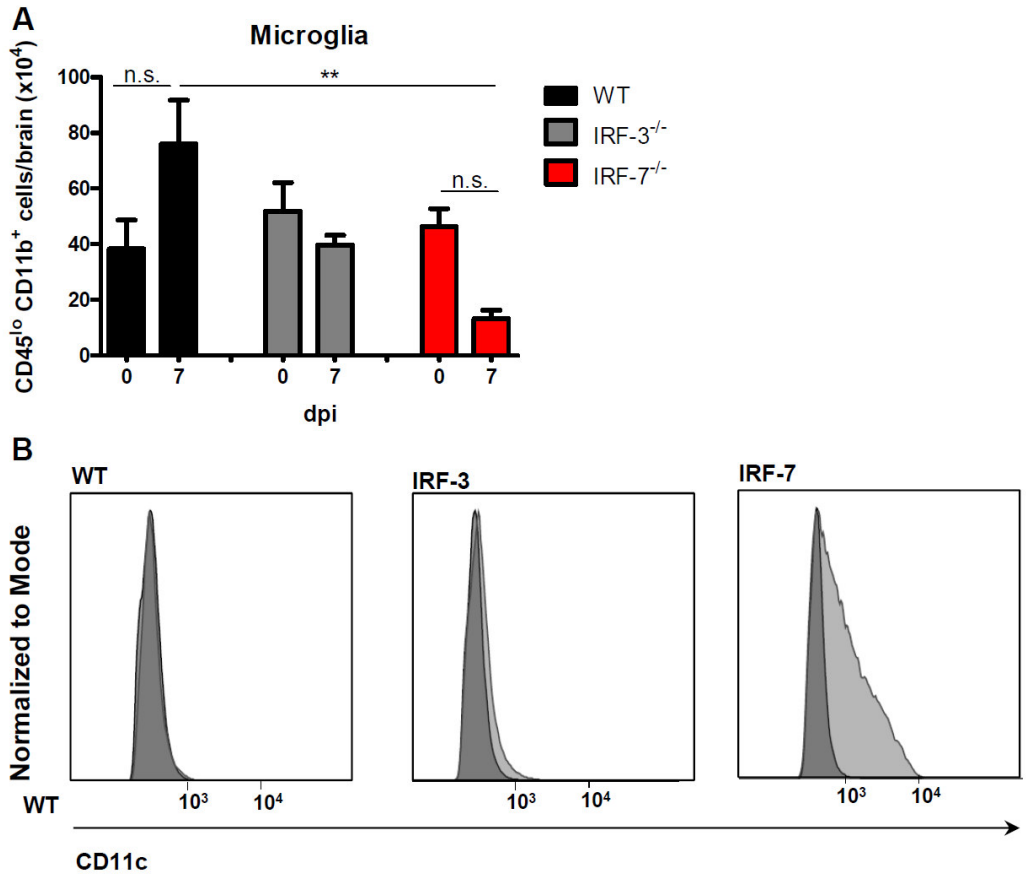

Fig.23: Alterations in IRF deficient microglia in the infected CNS. $CD45^{lo}$ and $CD45^{int}$, $CD11b^{+}$ Microglia were isolated from brains of uninfected and systemically LGTV infected WT, IRF-$3^{-/-}$ and IRF-$7^{-/-}$ (n=5), 7dpi. (A) Total number of microglia from isolated brain cells. (B) Microglia activation was determined by CD11c staining. Histograms show an overlap of uninfected populations (dark grey) with infected (light grey) populations. Statistical analysis was performed by 1way ANOVA, *** $p<0.0001$. Standard error of mean is depicted (SEM).

LGTV infection in WT mice leads to an increase in microglia numbers whereas quantities are not changed in IRF-3 deficient mice. Interestingly, in IRF-7 deficient mice microglia number decreases, indicating a special role of IRF-7 in proliferation or cell death of microglia. Upon activation microglia increase expression of CD11c on the cell surface [156]. To determine changes in activation status of microglia we determined CD11c and CD45 expression of microglia. WT microglia did not show an increase in CD11c expression. However, CD11c expression on microglia of IRF-$7^{-/-}$ mice increases upon infection. This indicates that although the numbers of microglia decrease upon infection, these cells show an activated phenotype.

Alterations in the activation profiles of immune cell populations are already known to mediate viral clearance but also neuropathological effects [122]. To evaluate if the

activation of immune cells is a general alteration in the neurotropic immune response of IRF-7$^{-/-}$ animals to LGTV, different infiltrating immune cell populations were analysed according to their activation profile by CD80+ expression (Fig.24).

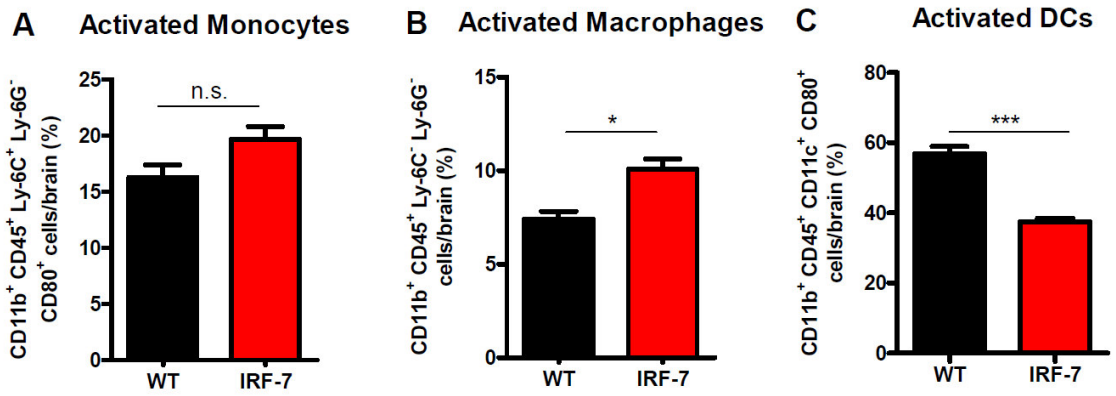

Fig.24: Activation profiles of infiltrating immune cells in the absence of IRFs. Monocytes (A), macrophages (B) and DCs (C) were isolated from systemically infected (7dpi) WT (n=5) and IRF-7$^{-/-}$ (n=3) mice. Cells were characterized as CD80^{+}CD11b^{+}Ly-6C^{-}Ly-6G^{+} monocytes (A), CD80^{+}CD11b^{+}Ly-6C^{-}Ly-6G^{-} macrophages (B) and CD80^{+}CD11b^{+}CD11c^{+} DCs (C) in % of the whole population. Statistical analysis was performed by 1way ANOVA, *** p<0.0001. Standard error of mean is shown (SEM).

No differences in the activation profile of monocytes were detected between WT and IRF-7$^{-/-}$ (A). A significantly higher number of activated macrophages was found in the IRF-7$^{-/-}$ mice compared to WT (B).
Interestingly, more activated DCs were found in WT controls compared to IRF-7$^{-/-}$ (C). This indicates that the absence of IRF-7$^{-/-}$ leads to a different activation pattern in infiltrating immune cells of the CNS during infection. It is not clear if these differences might be strong enough to have an impact on overall inflammatory processes or the efficiency of viral clearance in the CNS.

Immune cell infiltration and activation is mediated by pro- and anti-inflammatory cytokines. To assess if viral replication induces pro-inflammatory cytokine TNF-α and the immune cell attractant CCL2, expression levels were determined by RT-PCR (Fig.25) [17].

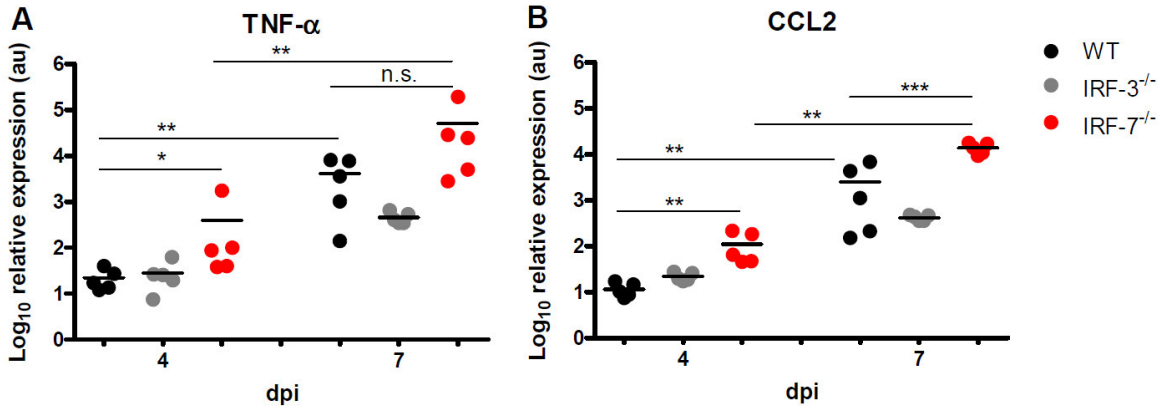

Fig.25: Alterations in inflammatory cytokine levels in the CNS upon LGTV infection. Mice were systemically infected with 1x 10^4 FFU LGTV. Expression of the inflammatory cytokines TNF-α (A) and CCL2 (B) at 4 and 7 dpi were determined in the olfactory bulb of the brain by quantitative real time RT-PCR. Expression was normalized to β-Actin as housekeeping gene and relative expression determined based on uninfected WT samples. Statistical analysis was performed by 1way ANOVA, *** p<0.0001.

Due to high viral levels in different brain parts (see Fig. 19 and 20), the olfactory bulb was chosen as a representative region to test alterations in different cytokine levels induced by virus infection. TNF-α (A) was clearly upregulated in the course of infection, independent of the genotype of the animals. However, in IRF-$7^{-/-}$ animals a significant increase was detected already on day 4 pi compared to WT and IRF-$3^{-/-}$. The expression levels of TNF-α rose even further until 7 dpi. The same trend was observed for CCL2 expression (B). CCL2 expression increased during the infection in all animals but the loss of IRF-7 led to higher increased expression levels.

This shows that a higher inflammatory response represented by high CCL2 and TNF-α expression levels can be seen in the CNS of WT and IRF-3 but especially IRF-7 deficient animals. This might be due to higher viral levels in IRF-$7^{-/-}$ animals (Fig.20), higher influx of immune cells into IRF-$7^{-/-}$ brains (see Fig. 22) or can be an intrinsic mechanism of the local loss of IRF-7 in the CNS. These differences have been detected in the olfactory bulb but differences in other brain regions are likely, since many cytokines exhibit long distance properties [157].

Viral replication and cytokine mediated effects can cause enhanced cell death upon infection. This is especially critical in the mammalian CNS where most of the neuronal populations are irreplaceable by the adult host. We found increased viral replication, elevated TNF-α level and enhanced numbers of $CD8^+$ T cells in the CNS of infected IRF-$7^{-/-}$ animals. These components are known to influence inflammation associated

processes and cell death [158]. Therefore the aim was to determine if apoptosis is enhanced in the brain of IRF-7$^{-/-}$ animals compared to WT, IRF-3$^{-/-}$ and IPS-1$^{-/-}$ mice (Fig.26).

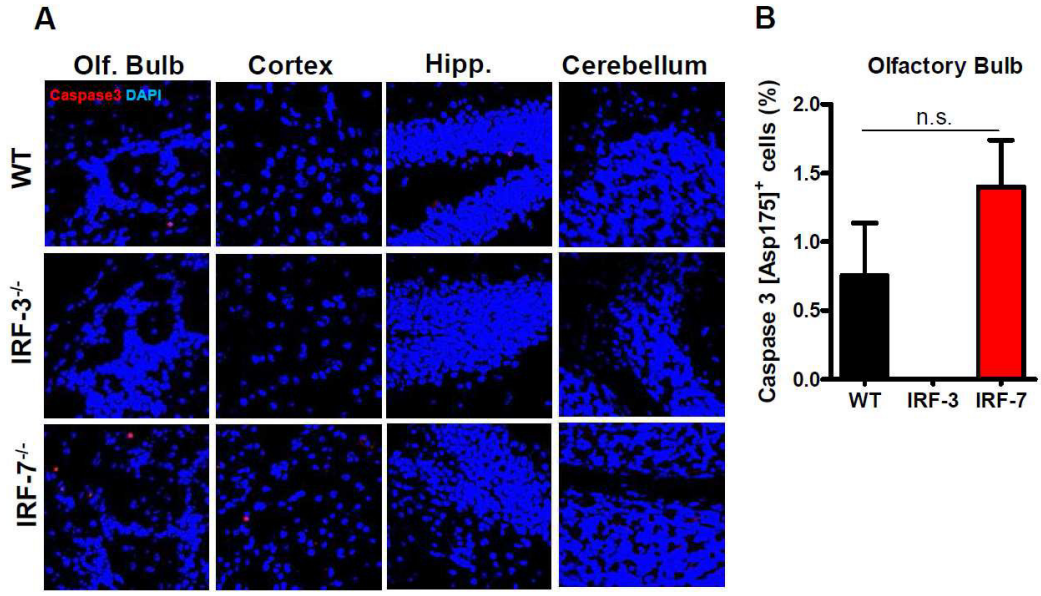

Fig.26: The influence of type I IFN deficiencies on apoptotic processes in the CNS in viral infection. Brains of systemically infected WT, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and IPS-1$^{-/-}$ mice (7dpi) were analysed for active Caspase 3 (Asp 175^{+}) positive cells. Nuclei of cells were stained for DAPI. (A) Displayed are representative 30 µm IHC sections of olfactory bulb, cortex, hippocampus and cerebellum. Magnification 40x and scale bar is indicated (50 µm). Caspase 3 positive cells were calculated for the OB to the number of DAPI positive cells in (%) of n≥5. Statistical analysis was performed by 1way ANOVA, *** p<0.0001. Standard error of mean is shown (SEM).

In different brain regions apoptotic cells could be detected for all genotypes upon infection with LGTV (A). Nearly no apoptotic cells were detected in uninfected animals (data not shown). In WT only few apoptotic cells could be observed in the olfactory bulb and none in other brain regions. The CNS of IRF-3$^{-/-}$ mice showed no apoptotic cells at all. In contrast, Caspase 3 positive cells were detected in all brain regions of IRF-7$^{-/-}$ and IPS-1$^{-/-}$ animals. Since the highest number of apoptotic cells was observed in the olfactory bulb differences were quantified in this region (B). The number of apoptotic cells in the olfactory bulb increased from 1 % in WT mice to approximately 3 % in IPS-1$^{-/-}$ animals. Only IPS-1$^{-/-}$ animals showed a significant increase of apoptotic cells in the CNS. Still, the overall number of apoptotic cells even in the brain of IPS-1$^{-/-}$ mice was comparably low and probably not sufficient to explain enhanced mortality in these animals. Therefore enhanced inflammatory responses do not contribute to Caspase 3 mediated cell death.

2.3.3. Cellular tropism in LGTV infection in the absence of IRFs

LGTV and TBEV target especially neuronal cells in humans and mice [20][159]. However, the loss of type I IFN signalling components has often been shown to lead to altered tissue tropism upon infection [160,161]. For this reason the tissue tropism of LGTV for different brain resident cells was analysed in detail (Fig.27).

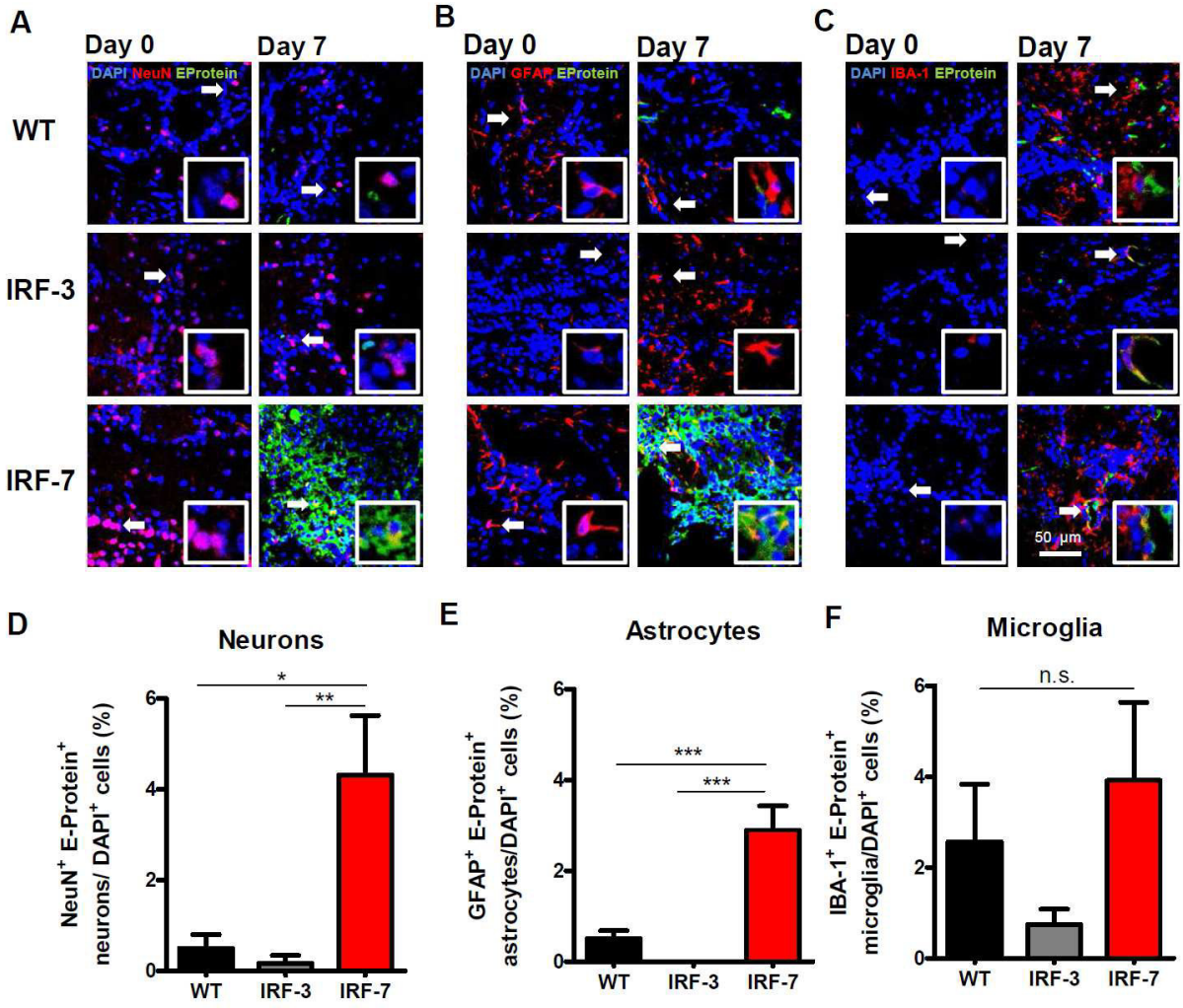

Fig.27: Altered tropism of LGTV to brain resident cells in the absence of IRF-7. IHC of the olfactory bulb of systemically LGTV infected WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice (n≥3). Neurons were identified as NeuN^{+} (A), astrocytes as GFAP^{+} (B) and microglia as IBA-1^{+} (C) cells and nuclei counterstained with DAPI. LGTV was detected by viral E-Protein. Pictures were taken at 40x magnification and scale bar is indicated (50 µm). White arrows indicate magnified infected and uninfected neurons, astrocytes and microglia at 0 and 7 dpi. The number of infected cells was quantified and normalized to DAPI^{+} cells. Data is shown for neurons (D), astrocytes (E) and microglia (F). Statistical analysis was done by 1way ANOVA, *** p<0.0001. Standard error of mean is depicted (SEM).

The olfactory bulb was chosen as representative region for the analysis of infected brain resident cells. In the WT situation neurons, astrocytes and microglia can be infected in relatively low numbers. In contrast, the loss of IRF-7 led to a significant increase of infected brain resident cells compared to WT and IRF-3$^{-/-}$ (D-F). Neurons are highly infected by LGTV which is according to literature (D). However, the neuronal marker NeuN failed to stain specifically periglomerular neurons of the glomerular cell layer that is mostly affected by LGTV infection. Therefore the number of infected neuronal cells is expected to be much higher than displayed here. Astrocytes that lack IRF-7 are also highly susceptible to LGTV whereas nearly no infected astrocytes can be detected in WT and IRF-3$^{-/-}$ animals (E). The number of infected microglia varies substantially between representative regions of the olfactory bulb and therefore no significant differences can be determined between different genotypes (F).
Overall, a shift in the cellular tropism towards the infection of astrocytes was determined for IRF-7$^{-/-}$ deficient animals additionally to the infection of neurons. This implies a locally defined role of IRF-7 in the infection of brain resident cells and specifically in astrocytes.

2.4. Local protective roles of IRFs in LGTV mediated CNS infection

Systemic infections lead to broad immune responses that might overlay or mask organ specific local peculiarities [20]. For instance the massive influx of leukocytes into the CNS upon infection and their cytokine mediated side effects might counteract potential anti-inflammatory responses that are elicited by neuroparenchymal cells themselves. To disassemble and evaluate specific LGTV mediated effects on cells of the CNS a local infection of brain cells and the analysis of specific resident cell types is needed.

2.4.1. Local LGTV infection in the CNS of IRF deficient animals

Even though it is assumed that LGTV enters the CNS after systemic infection, local and regional differences can be determined more easily by infections that are locally restricted. Intra cranial (i.c.) infections can provide more detailed information about brain intern restriction mechanisms and were therefore employed here, to determine differences in the susceptibility of different genotypes to LGTV infection (Fig. 28).

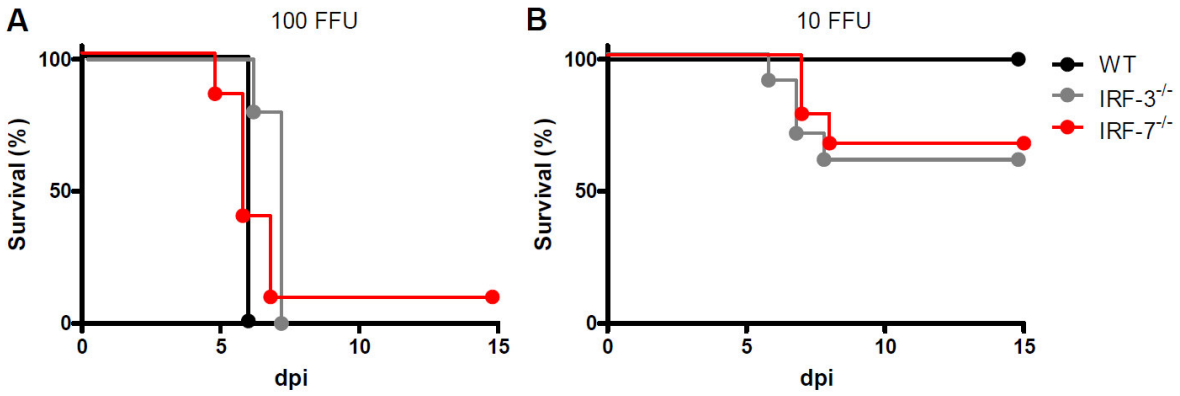

Fig.28: Impact of distinct IRFs on local LGTV infection. WT (n≥5), IRF-3$^{-/-}$ (n≥5) and IRF-7$^{-/-}$ (n≥9) mice were infected intra cranially with 100 FFU (A) or 10 (FFU) LGTV. Survival of these animals was monitored for 15 days after infection.

In contrast to systemic infection (see Fig. 5) animals of all genotypes succumb rapidly to the local infection within 7 days post infection (A), even though the virus concentration was much lower compared to i.p. injection. This indicates a specific susceptibility of the CNS to LGTV infection. A ten times lower virus dose (B) rescued all WT animals, but only approximately 60 % of the IRF deficient animals. This data shows that IRF-3 and IRF-7 do have protective local roles in the CNS but this protection is clearly dependent on the viral burden. Here, no differences between IRF-3 and IRF-7 could be detected, which implies equally important roles in the restriction of initial viral replication in the CNS.

2.4.2. Cell type specific roles of IRF-3 and IRF-7 in the CNS upon LGTV infection

Brain resident cells play a specific role in the defence of invading pathogens. Neuronal cells, astrocytes and microglia have been shown to tightly regulate their immunomodulatory environment to reduce inflammation mediated damage to non- or slow proliferating cells [162,163]. However, these cells also take actively part in their own defence by the production of type I IFNs, antiviral factors and growth factors which promote their survival [164,165]. To determine the role of neurons, astrocytes and microglia, cells were tested *in vitro* for their susceptibility to LGTV and their ability to mount type I IFN responses in the absence of IRFs.

Since neuronal cells were highly infected in the *in vivo* mouse model, we determined viral replication in primary neurons (Figure 29).

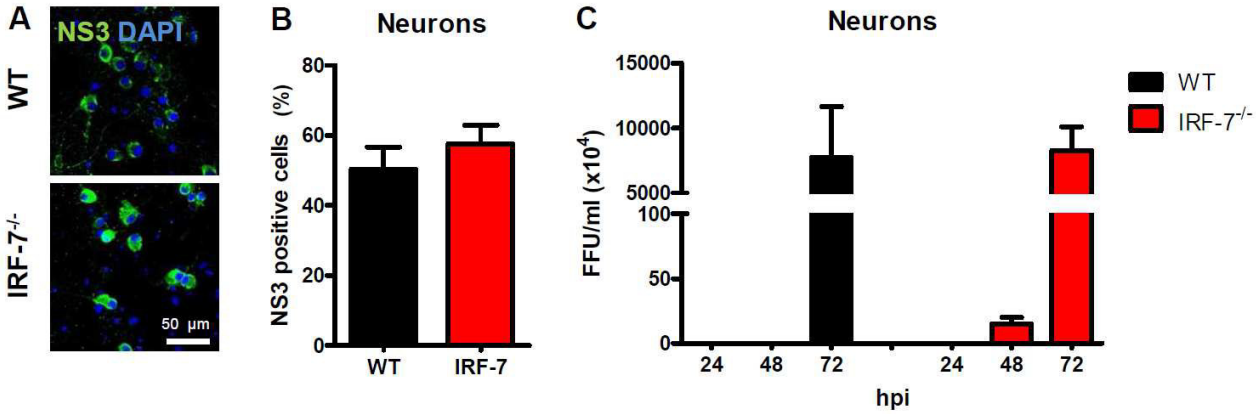

Fig.29: LGTV Infectivity and viral replication in neurons in the absence of IRF-7. Primary hippocampus derived neuronal cells from WT and IRF-7$^{-/-}$ animals were differentiated, cultured and infected with LGTV, MOI 0.001. (A) Cells were fixed and stained for viral antigen NS3 and nuclear DNA with DAPI (n≥5). Shown are representative pictures of neuronal cultures. Pictures were taken at magnification 40 x and scale bar is indicated (50 µm). (B) The number of infected cells was calculated to total DAPI positive cells in (%). (C) Infectious viral particles in the supernatants of cultures 24, 48 and 72 hpi were determined by FFU Assays. Statistical analysis was done by 1way ANOVA. Standard error of mean is shown (SEM).

Neuronal cells are highly susceptible to LGTV *in vitro* and more than 50 % cells are infected 72 hpi (A, B). No differences could be determined between WT and IRF-7$^{-/-}$. Similarly the amount of infective virus particles was quantified (C). Even though viral levels increase over time again no difference were seen comparing both genotypes. This differs from the *in vivo* situation (Fig.28). More IRF-7$^{-/-}$ neurons were infected which is probably due to enhanced viral replication in the CNS of IRF-7$^{-/-}$ animals *in vivo*. *In vitro* hippocampal neuronal cells from WT and IRF-7$^{-/-}$ are equally sensitive to infection with LGTV with similar kinetics.

Even though cells can be comparably well infected, the antiviral response in IRF-7$^{-/-}$ neurons might differ. Due to the missing feedback loop for type I IFN amplification which is mediated by IRF-7, the ability of these cells to secrete IFN might be impaired. Therefore the secretion of type I IFNs was analysed in neurons upon infection (Fig. 30).

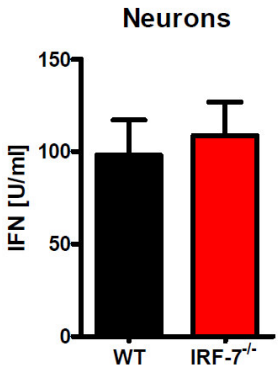

Fig.30: Type I IFN response in infected neuronal cells in the absence of IRF-7. Supernatants of WT and IRF-7$^{-/-}$ hippocampal neurons infected with LGTV (MOI 0.001) were tested in a bioassay for secretion of type I IFNs, 72 hpi (n≥5). IFN was determined as Units per ml. Statistical analysis was done by 1way ANOVA and standard error of mean is shown (SEM).

WT Neurons were able to produce type I IFN upon infection with LGTV. Although IRF-7 is responsible for the positive amplification loop in IFN induction no differences were detected between WT and IRF-7$^{-/-}$ cells. This indicates that IRF-7$^{-/-}$ neuronal cells can induce type equal amounts of type I IFN in an IRF-7 independent manner. Thus, even though neuronal cells are highly infected, they do not contribute to differences in infection that were seen in the *in vivo* model.

Other brain resident cells might intervene decisively in the infection process. Astrocytes for instance are known to contribute to the protection of neuronal cells by type I IFN induction and growth factor secretion [166]. Since also a higher number of astrocytes were infected *in vivo*, these cells might be affected by the loss of IRF-7. First of all, the response of these cells to LGTV infection was tested (Figure 31).

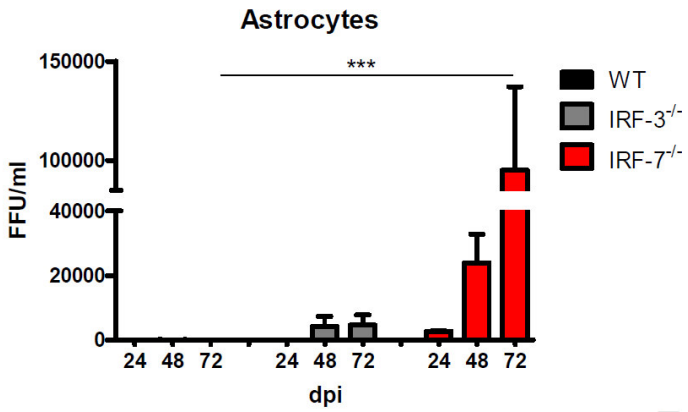

Fig.31: Susceptibility of astrocytes to LGTV infection in the absence of IRFs *in vitro*. Primary WT, IRF-3$^{-/-}$ and IRF-7$^{-/-}$ Astrocytes were infected with LGTV, MOI 0.1. Viral particles in supernatants were tested in the FFU Assay for infective virus particles at 24, 48 and 72 hpi (n≥5).

The statistical analysis was performed with 1way ANOVA, *** p<0.0001 and the standard error of mean is applied (SEM).

Nearly no infectious virus particles could be determined in LGTV exposed WT astrocytes. Whereas IRF-3 deficient astrocytes show only low level of viral replication, astrocytes which lack IRF-7 show an high increase of viral replication during the course of infection. This indicates that viral replication is effectively restricted by IRF-7 in astrocytes or that cells are more susceptible to initial infection. Thus, a specific role for IRF-7 in the protection of glia cells during LGTV infection is implicated.

IRF-7 plays a role in the amplification of type I IFNs. We therefore determined the type I IFN response in these cells (Figure 32).

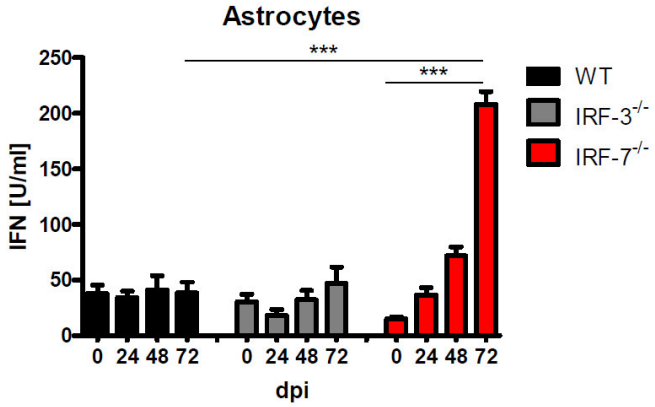

Fig.32: Elevated type I IFN response in infected astrocytes in the absence of IRF-7. WT, IRF-$3^{-/-}$ and IRF-$7^{-/-}$ astrocytes were infected with LGTV, MOI 0.1 and supernatants of uninfected and 24, 48 and 72 hpi tested for type I IFN responses in a reporter assay ($n \geq 5$). The statistical analysis was done by 1way ANOVA, *** p<0.0001. Standard error of mean is shown (SEM).

WT astrocytes show higher basal IFN levels in the uninfected state compared to IRF-$3^{-/-}$ and IRF-$7^{-/-}$ cells. This indicates that astrocytes might be pre-activated and therefore able to induce faster immune responses. However, very low number of infective virus particles were found in WT astrocytes at all tested time points after infection (Fig.31). This suggests low viral replication induces only a very low type I IFN levels in WT astrocytes. The same correlation between low viral replication and low induction of type I IFN was found in IRF-3 deficient astrocytes. Surprisingly, despite the defective positive amplification loop depending on IRF-7, high amounts of type I IFN are secreted by IRF-$7^{-/-}$ astrocytes. This indicates that type I IFN can be induced in astrocytes in an IRF-7 independent manner (Fig.32).

The high type I IFN production by IRF-7 deficient astrocytes could be due to the high abundance of PAMPs and thereby increased triggering of the early type I IFN response,

or by the ability of the astrocytes to produce type I IFNs. To further investigate this, astrocytes were transfected with polyinosinic-polycytidylic acid (poly I:C) which is used as a synthetic analogue of dsRNA. The type I IFN induction was compared between WT and IRF-7$^{-/-}$ astrocytes (Fig.33).

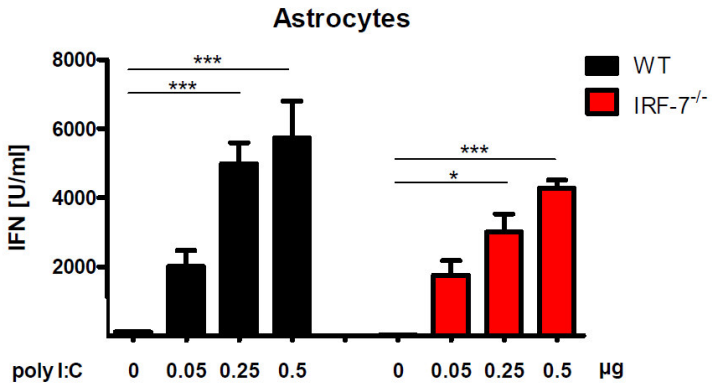

Fig.33: Poly I:C stimulated type I IFN production by IRF-7 deficient astrocytes. WT and IRF-7$^{-/-}$ astrocytes were transfected with 0.05, 0.25 and 0.5 µg poly I:C or left untreated (n=3). Type I IFN was measured by bioassay at 24 hours post transfection (hpt). Statistical analysis was done by 1way ANOVA, *** $p<0.0001$. Standard error of mean is shown (SEM).

Poly I:C induces a concentration dependent induction of strong type I IFN response in astrocytes . The response of WT and IRF-7$^{-/-}$ astrocytes is dependent on the amount of poly I:C that was deployed. However, no significant differences between WT and IRF-7$^{-/-}$ astrocytes were detected. This indicates that the ability of these astrocytes to produce type I IFN is dependent on the amount of PAMPs. Thus the stronger IFN response in IRF-7$^{-/-}$ astrocytes results from the higher susceptibility to the virus infection, with is associated with higher amount of PAMPs. In addition this verifies an IRF-7-independent induction of type I IFN response in astrocytes.

Microglia, might also contribute to a protective type I IFN response next to astrocytes as shown for other infections [167]. Therefore microglia were tested for type I IFN secretion *in vitro* as well (Figure 34).

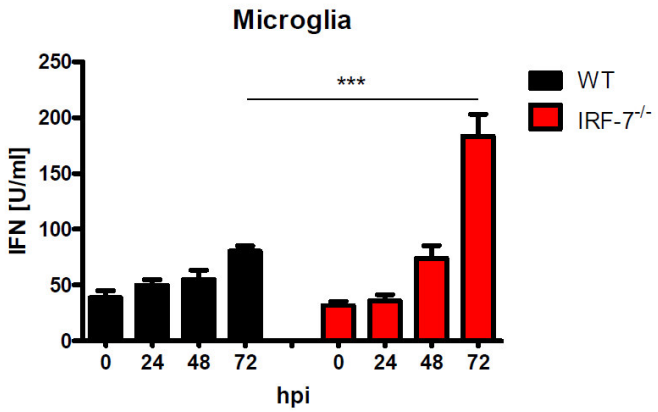

Fig.34: Type I IFN response elicited by infected microglia in the absence of IRF-7. WT and IRF-7$^{-/-}$ microglia were separated from mixed glia cultures, cultivated and LGTV infected with an MOI of 0.1. Secreted type I IFN was determined by reporter assay and is depicted as Units/ml (n=6). Statistical analysis was done by 1way ANOVA, *** $p<0.0001$. Standard error of mean is shown (SEM).

Increasing amounts of type I IFNs are produced by WT microglia during the course of LGTV infection. A similar rise was also seen in IRF-7$^{-/-}$ microglia, however significantly higher than in WT. This shows that also microglia were able to elicit high IFN responses in the absence of IRF-7 similar to astrocytes. Still, microglia cultures are often heterogeneous due to the isolation process and might still contain astrocytes which also contribute to the type I IFN production.

2.4.3. Type I IFN responses to neurotropic LGTV in the absence of IRFs

The induction of inflammatory factors such as type I IFN is dependent on the interplay of several different cell populations *in vivo*. To evaluate if higher type I IFN responses can be verified in systemic infections, IFN-reporter mice (IFN-β$^{+/\Delta luc}$) and IFN-β$^{+/\Delta luc}$ mice deficient for IRF-3 and IRF-7 were used for *in vivo* imaging (Figure 35).

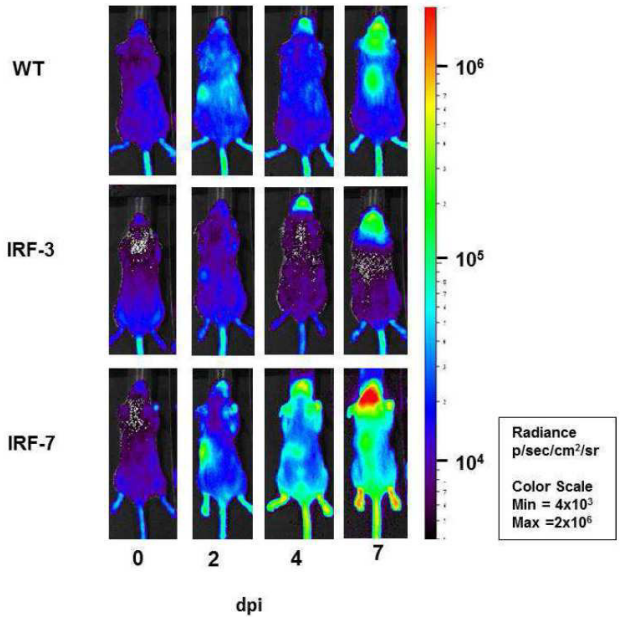

Fig.35: Type I IFN induction visualized in a LGTV infected IFN-β$^{+/\Delta luc}$ reporter mouse model. IFN-β$^{+/\Delta Luc}$, IFN-β$^{+/\Delta Luc}$ IRF-3$^{-/-}$ and IFN-β$^{+/\Delta Luc}$ IRF-7$^{-/-}$ mice (n≥3) were infected i.p. with 1x 10^4 FFU LGTV. Subsequent to *in vivo* application of luciferin, uninfected and infected mice were monitored 0, 2, 4 and 7 dpi during infection in the IVIS system. Representative mice of each group are displayed here in a dorsal position. The scale indicates the number of photons (p) measured per seconds (s) per cm^2 per steradian (sr).

In vivo imaging reveals an increase in type I IFN induction in the course of LGTV infection until 7 dpi in IFN-β$^{+/\Delta Luc}$, IFN-β$^{+/\Delta Luc}$ IRF-3$^{-/-}$ and IFN-β$^{+/\Delta Luc}$ IRF-7$^{-/-}$ mice. A much stronger signal was seen in IRF-7$^{-/-}$ animals compared to WT and IRF-3$^{-/-}$ mice. IFN-β expression in WT and IRF-3$^{-/-}$ mice is restricted to the CNS and the spinal cord, whereas robust IFN levels were detected in the CNS and the periphery of IRF-7$^{-/-}$ mice. This peripheral type I IFN induction that was seen in lymphoid organs and lung in earlier experiments (Fig. 8 and 9). Whereas in the periphery the producing cell type is not known, increased IFN-β expression in the CNS is attributable to IFN producing microglia and astrocytes in the absence of IRF-7.

Considering the correlation of type I IFN induction to viral levels, the IFN induction was checked for different brain parts. Since the viral load was highest in the olfactory bulb, the IFN induction in this region should be the highest (Figure 36).

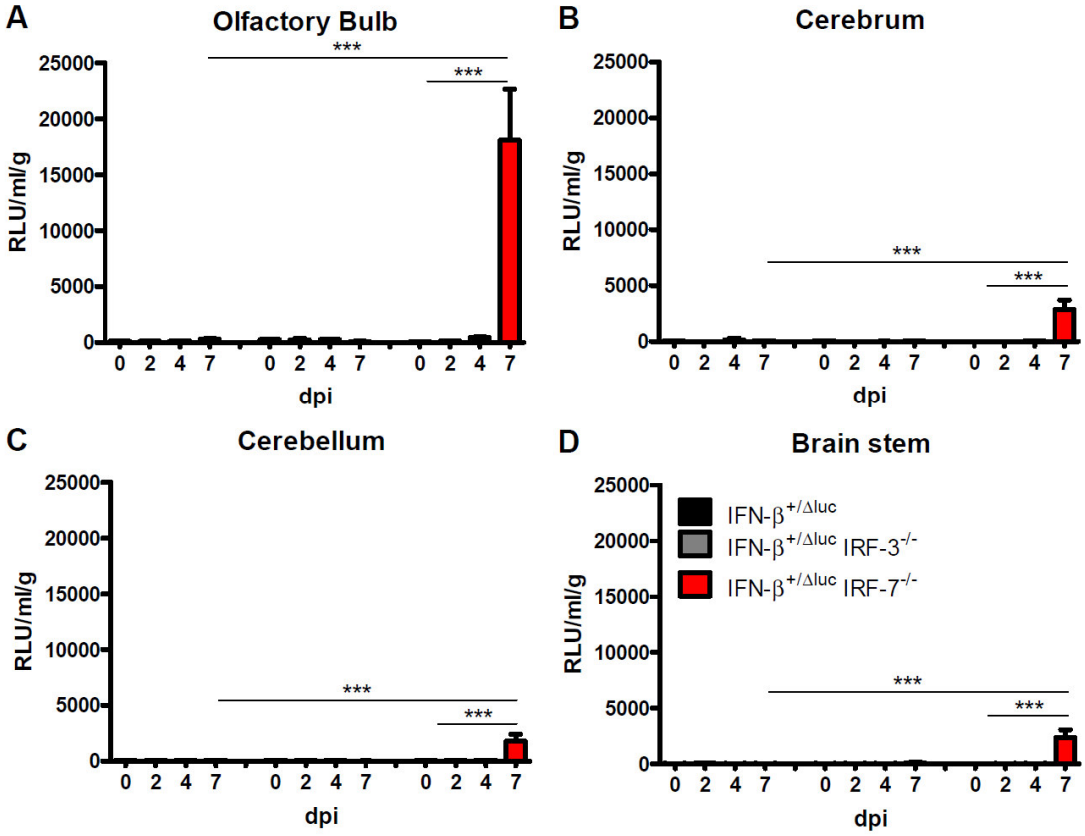

Fig.36: Type I IFN induction is highest in the olfactory bulb of IFN-β$^{+/\Delta luc}$ IRF-7$^{-/-}$ reporter mice. Brains of systemically with LGTV infected IFN-β$^{+/\Delta Luc}$, IFN-β$^{+/\Delta Luc}$ IRF-3$^{-/-}$ and IFN-β$^{+/\Delta Luc}$ IRF-7$^{-/-}$ mice (n≥5) were isolated and cell extracts of olfactory bulb (A), cerebrum (B), cerebellum (C) and brain stem (D) were checked for luciferase activity and represented as RLU per ml and normalized to 1 g of tissue. Statistical analysis was done by 1way ANOVA. Standard error of mean is shown (SEM).

Very low type I IFN induction was found in WT animals. Even though WT and IRF-3$^{-/-}$ animals are able to mount protective type I IFN responses in LGTV infection *in vivo*, the signal of IRF-7$^{-/-}$ mice is significantly higher in all tested brain parts, 7 dpi. Corresponding to viral load, type I IFN levels are strongest in the olfactory bulb (A). This shows once more that enhanced viral replication induces stronger type I IFN expression in the brain of IRF-7$^{-/-}$ animals.

2.4.4. Viral clearance in the absence of IRFs

Increased viral levels in the absence of IRF-7 elicit enormous type I IFN production which is induced at high expenses even without relying on the IRF-7 positive feedback loop (A-D). This raises the questions about the effectiveness of viral clearance from the CNS. Is

type I IFN sufficient or does the loss of IRF-7 affect the course of infection beyond the acute phase? This question was addressed by the validation of viral presence in the CNS when animals appear again healthy and body weight returned to the level of recovered WT animals (see Fig.6). Results are shown in Figure 37.

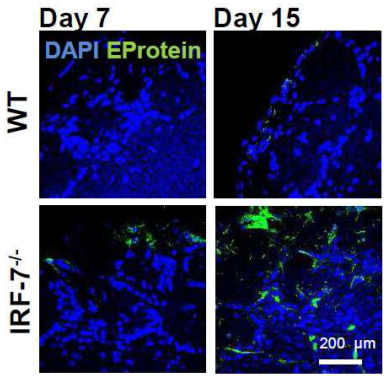

Fig.37: In the absence of IRF-7, LGTV is able to persist in the brain. WT and IRF-$7^{-/-}$ mice were infected i.p. with 1x 10^4 FFU LGTV and organs isolated 7 and 15 dpi. Brains were fixed and embedded and 30 µm sections used for IHC analysis. Brain sections were stained for viral E protein and nuclear cells by DAPI. Pictures of the olfactory bulb were taken at a magnification of 20x and the scale bar is indicated (200 µm).

Few viral signals can be detected in the olfactory bulb of infected WT animals 7 and 15 dpi. In contrast, a clear virus signature can be shown in the olfactory bulb of IRF-$7^{-/-}$ animals 15 dpi. This shows that even though higher type I IFN levels were elicited, this was not sufficient to clear the virus from the CNS. Even after the acute phase of infection, the virus can persist in the brain of IRF-$7^{-/-}$ animals.

3. Discussion

Innate immunity ensures a fast response against invading pathogens and coordinates the activation of the adaptive immune response [27]. This defence is mediated on several levels by interlocking mechanisms, such as the complement system, the production of cytokines and the engagement of cellular components [42][168,169]. Ideally this results in a reduced propagation of the pathogen and subsequent clearance [170]. However, defects in the innate immune response often lead to a more widespread dissemination of the pathogen, the involvement of different organs and subsequently, a functionality loss [20][171]. This might ultimately result in death of the host [71].

3.1. LGTV recognition by RLRs and TLRs is crucial for survival

The type I IFNs plays a crucial role in the control of viral infections [20][151]. Viral recognition by cytosolic receptors such as RIG-I and MDA5, as well as endosomal receptors like TLRs have been shown to protect the host by the activation of the type I IFN signalling pathway [151][172]. Type I IFNs induce the expression a broad range of defence molecules which regulate cellular mechanism such as cell survival, proliferation and secretion of other inflammatory factors [44][52].

LGTV, a virus of the TBEV serogroup, is able to evade early innate immune recognition and interferes with type I IFN production in the host [132][173]. Still, hosts with a functional immune are able to clear systemic infections [20].

Defects in viral recognition and type I IFN production, such as the loss of IFNAR, led to viral replication and systemic spread of the virus which cannot be controlled by the host [20]. In contrast, the loss of IFN-β, a type I IFN, did not result in diminished survival in mice [20]. This indicates that the function of some signalling components can be compensated whereas others are crucial for survival.

Further components of the type I IFN induction pathway are needed for viral defence in LGTV infection. TRIF, which forwards signals of viral recognition by TLR3, was not needed for mouse survival of LGTV infection. Still, deficiencies in TLR3 signalling might play a less obvious role in antiviral defences as it was shown in other viral infections [174]. A study on human patients also indicated a role for TLR3 in TBEV infection [126]. These differences might be explained by variations in the pathogenicity of TBEV strains compared to the attenuated LGTV strain TP21 [20][175].Differences in the innate immunity between the human and the rodent system have to be considered as well [111][176]. It was shown that TLR3 is not only differentially expressed on murine and

human immune cells but the receptors react differently to pathogenic stimuli like LPS or the synthetic analogous of double stranded RNA, poly I:C [176–179].
Myd88 which functions as adaptor molecule for endosomal TLRs such as TLR7 and TLR9 was indispensable for survival. A similar important role was determined for RIG-I and MDA5 adaptor molecule IPS-1. Therefore both, endosomal and cytosolic virus recognition is needed in the defence of LGTV. Cytosolic sensors were needed for early recognition, whereas TLR7 and TLR9 mediated signalling led to a delayed onset of symptoms. Since it is known that TBEV is able to hide initial replication in the endoplasmic reticulum, a similar mechanism might be proposed for LGTV [180]. Viral material is probably not only secreted into the cytosol, but might also be transported from the ER through the endomembrane system of the Golgi apparatus to endosomes. This might provide an explanation for the late recognition of virus RNA by endosomal TLRs.
Differences in the time line for TLR and RLR signalling might also reflect a specific role of these receptors in the periphery or the CNS upon infection. It is known that type I IFN induction in the periphery controls viral replication. A delayed peripheral IFN response might facilitate initial viral replication and early spread to the CNS where the virus can not be controlled [20]. Differences in RLR and TLR subsets have also been noted in different brain parts and might therefore contribute to pathogenesis. A specific role of RLR adaptor molecule IPS-1 has been shown for IFN-β expression in the olfactory bulb whereas other regions were unaffected [151]. Here, other recognition receptors like TLRs might compensate efficiently for the loss of RIG-I and MDA-5 signalling.

3.2. Diverse roles of interferon regulating factors in LGTV infection

Interferon regulatory factors are important to initiate and fine tune the type I IFN response upon infection [181]. Whereas IRF-3 plays merely a role in early type I IFN induction, mediated by IFN-β, IRF-5 is known to activate the inflammatory NF-κB pathway [31][182]. IRF-7 functions as a positive regulator for a reinforced type I IFN response characterized by increased IFN-α levels [71]. It was shown that these proteins induce overlapping but also distinct antiviral gene sets [183]. It was proposed that IRF-3 and IRF-7 have no redundant roles [69]. In this context, infections with different viruses such as WNV, EMCV and HSV-1 were lethal in the absence of IRF-3, IRF-5 or IRF-7 [54][71][184]. However, distinct virus infections revealed individual roles of these proteins independent of viral concentration and route of infection [31][54][184]. Mice deficient for IRF-3 survive HSV-1 infection, whereas the loss of IRF-7 led to death [71]. In WNV infection, the absence of either factor was associated with a lethal outcome [54][185]. The role of IRFs in LGTV infection was not known so far. Here it was shown that in systemic LGTV infection, IRF-3

and IRF-7 were not essential for survival, whereas the loss of IRF-5 led to 50 % mortality in mice. Interestingly, local LGTV infection showed a very different outcome. In the CNS survival was dependent on viral quantities. Intermediate viral infection doses led to high mortality rates in all animals. In contrast, very low quantities of LGTV were tolerable for WT but still lethal for most IRF-3 and IRF-7 deficient animals. This indicates to a specific role of IRF-3 and IRF-7 in the CNS during LGTV infection.

3.3. IRFs are protective in peripheral LGTV infection

Even though the loss of IRF-7 did not affect survival, a temporary weight loss indicated a potential function of IRF-7 in LGTV infection. In other virus infection models, the absence of IRF-3 and IRF-7 was associated with higher viral loads and an increased spread of the virus which was now also verified for LGTV infection[54][185].

Here, LGTV infection caused an activation of the immune system, marked for instance by splenomegaly and lymph node hyperplasia. This is often a result of enhanced immune cell proliferation [186]. Increased numbers of macrophages, granulocytes and CD8 T cells were found in spleens of infected IRF-7$^{-/-}$ animals, without overall changes in the microscopic integrity of the organ. These cells are often associated with inflammatory processes and migrate to the site of acute infection [146].

Another sign for immune activation was IFN-β induction by LGTV infection. Systemic type I IFN was low in the absence of IRF-7, which was similar to results published for WNV, EMCV and HSV-1 [54][71]. The local IFN induction was highly elevated in several lymphoid and non-lymphoid organs of IRF-7$^{-/-}$ animals. This was surprising, since IRF-7 was postulated as the master regulator of type I IFNs, where a loss of the protein was associated with decreased type I IFN levels [71].

Especially myeloid immune cell populations, known as IPCs are able to secrete high amounts of protective type I IFNs upon infection [150]. It is known that these cells can be infected by LGTV [20]. Still, only low infection rates were observed in IRF-7$^{-/-}$ BMDCs but an increased susceptibility of IRF-3$^{-/-}$ cells was seen. This does not coincide with *in vivo* data from spleen where enhanced viral level for IRF-7$^{-/-}$ but not IRF-3$^{-/-}$ were detected. Therefore local factors and compensation mechanisms like the production of type I IFN might contribute to the protection of myeloid IRF-3$^{-/-}$ cells. In fact, differentiated bone marrow derived IRF-3$^{-/-}$ pDCs and macrophages showed nearly normal type I IFN induction whereas IFN secretion by IRF-7$^{-/-}$ cell was clearly impaired. This corresponds to data that shows that IFN production by pDCs is crucially dependent on IRF-7 [71]. Still, residual type I IFN secretion was observed in IRF-7$^{-/-}$ macrophages. In WNV infection, IFN-α level in IRF-7$^{-/-}$ macrophages were clearly decreased whereas the production of

IFN-β was less affected [53]. It can be speculated that enhanced IRF-3 activation leading to increased IFN-β expression might at least to some extent compensate for the loss of the positive feedback loop mediated by IRF-7. Still, this does not explain the high type I IFN induction that was seen in lymphoid organs in the absence of IRF-7.

To evaluate this phenomenon a more detailed *in vivo* analysis of the peripheral infection would be necessary. Myeloid IRF-$7^{-/-}$ cells might still be able to produce high IFN amounts depending on the infection stimulus. Also cell-cell interactions and environmental cues might influence the antiviral response. Compensatory functions of IRF-3 and IRF-5 might also contribute to the effect[187]. The absence of distinct IRFs can result in the induction of modified IFN-α subtypes profiles [27].

After all, *in vitro* BMDCs cultures might reflect poorly the systemic impact of LGTV on IRF-$7^{-/-}$ animals.

3.4. The role of type I IFN signalling in the CNS

The CNS was initially described as immune privileged site, characterized by relatively immune quiescence and only minor interaction with components of the immune system [188,189]. This was thought to minimize pathogen entry and reduce damage caused by activated immune cells in a system that relies on a functional neuronal network for survival [188,190,191]. However, in the last decades this hypothesis was seriously challenged [188]. Even though barriers like the BBB and the CSFB isolate the organ and control exchange of nutrients and signalling molecules, immune cells have been shown to enter the CNS actively to scan for foreign antigens [192–194]. Also brain resident cells were not as quiescent as initially assumed. Especially microglia and astrocytes take very actively part in local immune responses to protect neuronal integrity [195,196].

Neurons, microglia and astrocytes are able to produce type I IFNs locally in varying amounts upon different viral infections [165][169][197]. This local activation mediates the production of cytokines, antiviral factors and facilitates enhanced immune cell migration into the brain [17][151]. Still, this response is far from homogeneous. Different areas of the CNS are more accessible to immune cells and immune responses from brain resident cells differ substantially from each other [163][198]. Additionally, differences have been described for subpopulations of the same cell type and the maturation status of these cells [152][199].

3.5. The mechanism of CNS entry in LGTV infection

That LGTV is a neurotropic virus is well established, whereas the mechanism by which the virus enters the CNS is still unknown [20][151]. Due to experimental data, several routes of infection are imaginable and often a virus uses more than one entry strategy [122–124]. Here, defects in the type I IFN response seem to provide pathogens with an easier access to a more vulnerable CNS [20][151]. It was shown that the virus can already be detected early in the CNS before the inflammatory response leads to a breakdown of the BBB in IFNAR mice [20].

A possible entry strategy is the infection of immune cells, which might enable LGTV to enter the CNS undetected by a ´Trojan Horse mechanism´ [20]. Leukocytes which are defective in their antiviral response such as seen here for hematopoietic IRF-3$^{-/-}$ cells are more susceptible to LGTV infection and might provide a better target for LGTV. Infected infiltrating immune cells which continuously patrol the CNS might expose susceptible neurons to LGTV.

We also observed an early and transient viremia in the absence of IRF-7. For many different arboviruses a viremia has been described [121]. This might enable the virus to spread to different organs and ultimately pass the BBB to gain access to the CNS.

However, we could also show recently that in IPS-1$^{-/-}$ animals the virus locates especially to the glomerular layer of the olfactory bulb [151]. This might indicate an infection of olfactory sensory neurons in the periphery, which continue through the cribriform plate directly into the exposed olfactory glomeruli [200]. Further research is needed to verify if one or several of these entry routes are used by LGTV in systemic infection and which impact is due to specific defects in the type I IFN response.

3.6. IRF-7 protects the CNS from increased viral replication

Viral replication in the CNS can occur at the site of entry, but virus can also spread into different areas of the brain [200][201]. Viral recognition receptors like TLRs and RLRs which recognize PAMPs of LGTV are unequally distributed in different brain resident cells and regions of the CNS [202]. Some areas might therefore induce immune responses less efficiently than others and therefore the amount of inflammatory cytokines differs enormously [195]. In this context, the cytokine profile of cell populations by environmental cues is important. Cells of different origin are able to mount faster and more effective responses due to higher initial expression of type I IFN associated factors [152].

In the absence of IPS-1, lower levels of basal IRF-1 and viperin were observed in the olfactory bulb. This might explain increased replication of LGTV in this area [151].

Similar to LGTV infected IPS-1$^{-/-}$ animals the virus was detected here mainly in the glomerular layer of the olfactory bulb. This indicates a certain preference of LGTV for glomerular or sensory olfactory neurons. This might be due to regional differences in neuronal populations and their maturation status [152][199][203]. Neuronal precursors migrate from the hippocampus along the rostral migratory stream (RMS) into the olfactory bulb where mainly sensory neurons are continuously replaced [204]. It is not clear, if these cells show the same defence and maturation properties like stationary older neurons or if there are differences in their cytokine expression patterns. Less mature neurons were shown to be more vulnerable in other viral infections which might result in a preference of LGTV for the olfactory bulb [205]. However, regions with immature neurons, such as the hippocampus, were merely LGTV free [206]. No clear manifestation of the virus in other specific brain regions or structures of the CNS were detected.

Even though the localisation of the virus was similar in all genotypes, viral amounts were substantially dependent on type I IFN signalling. The loss of IRF-7 led to increased viral amounts in the olfactory bulb. This can be due to higher viral amounts in the periphery and subsequently enhanced infiltration of the virus into the CNS. In the absence of IRF-3, virus was nearly undetectable in the brain. This indicates that the loss of IRF-3 is negligible in systemic infection targeting the CNS, whereas IRF-7 function can not easily be compensated by other factors.

3.7. IRF-7 mediates inflammatory processes in the LGTV infected brain

Enhanced propagation of pathogens in the CNS is often associated with inflammation [151]. This process is for instance characterized by high cytokine production, microglia activation and immune cell infiltration [151]. Cytotoxic side effects can lead to neuronal cell death and are therefore tightly controlled [207]. The deficiency of IPS-1 in LGTV infection led to increased levels of IFN-β and IL-6, as well as an increase in chemokine levels [151].

Increased level of pro-inflammatory cytokine TNF-α and chemoattractant CCL2 were detected here in the olfactory bulb of all infected animals. The amounts increased during the course of acute infection and this effect was most pronounced in LGTV infected IRF-7$^{-/-}$ animals. In these mice the upregulated cytokine milieu was accompanied by a high influx of infiltrating immune cells into the CNS characterized as monocytes, macrophages, DCs and T cells.

These invading cells play diverse roles in the infected CNS. DCs act as antigen presenting cells and directly influence the number and type of T cells that are recruited during infection [208]. T cells can be polarized to a Th1 or Th2 fate which is associated

with different cytokine profiles [208]. Similar polarizations have also been described for macrophages, which can be termed inflammatory M1 or anti-inflammatory M2 cells [209]. Due to effector function and cytokine production these macrophages can support survival or mediate cell death [209]. Interestingly, more activated IRF-7$^{-/-}$ macrophages but less activated IRF-7$^{-/-}$ DCs have been characterized compared to controls. This might influence antigen presentation and therefore viral clearance as well as cell survival in the CNS of these animals. A similar activation was found in brain resident microglia. The overall number of these cells was reduced in the absence of IRF-7. However, cells showed increased activation. This might result in microglia conversion to DC like cells with improved antigen presenting properties [210,211]. Microglia can also switch to a more macrophage-like phenotype by increased CD45 expression in the inflamed CNS [156].
Interestingly, IRF-7 was shown to play a role in the M1 to M2 polarisation of microglia in the brain [212]. In the proposed model, transforming growth factor-beta (TGF-β) was able to reduce IRF-7 activity, which was associated with increased TNF-α levels and the retention of microglia in the M1 state [212]. Similarly, elevated levels of TNF- α were detected here in the absence of IRF-7 and macrophages as well as microglia show an activated phenotype. Therefore not only increased viral replication but also an IRF-7 dependent, CNS intrinsic mechanism might be responsible for increased inflammation in the brain of IRF-7$^{-/-}$ animals.
An increase in the inflammatory response by the lack of IRF-7 might affect cell viability in the CNS. LGTV infection was shown to enhance Caspase 3 mediated apoptosis in Neuro-2a cells *in vitro* which was at least partly mediated by the E Protein of the virus [131]. *In vivo* the loss of IPS-1 led also to increased apoptosis in the olfactory bulb [151]. No enhanced apoptosis was seen in the absence of IRF-7 upon LGTV infection despite elevated TNF-α levels and an increase of cytotoxic T cells. Therefore neuronal integrity might be largely maintained. However, cell death by caspase independent apoptosis, necrosis or autophagy was not explored and might play a role in LGTV infection [213].

3.8. IRF-7 affects regional and cell specific immunity in neurotropic infection

Often specific cell types of the CNS are targeted by pathogens. Not only neurons but also astrocytes can be highly susceptible to virus infections [214,215].
Neurons have been shown to be the major target of LGTV and TBEV infections in the CNS in humans and rodents [20][119]. This was confirmed here, since more than half of the differentiated hippocampal neurons were infected by low quantities of LGTV *in vitro*. *In vivo*, neurons in the olfactory bulb were also highly infected, especially in the absence of IRF-7. However, IRF-7$^{-/-}$ neurons were equally vulnerable to LGTV *in vitro* even though

higher infection rates were observed *in vivo*. This might be due to higher local viral replication in the CNS.

Additionally, properties of regional neuronal subpopulations might play a role. Neurons isolated from cerebellum and cortex did not show the same susceptibility to WNV infection, due to differing basal levels of components of the type I IFN signalling pathway [152]. Here, only hippocampal neurons were tested. *In vivo* the virus targets mainly glomerular and sensory neurons but not granule cells and mitral cells of the olfactory bulb. It can be hypothesized that IRF-7 plays a specific role in the protection of distinct neuronal subpopulations. Also other regional cell types like astrocytes and microglia might influence the neuronal susceptibility.

A clear switch in tissue tropism of LGTV was observed in astrocytes in the absence of IRF-7. Whereas these cells are normally sparsely infected, IRF-7$^{-/-}$ astrocytes were highly affected *in vivo* and *in vitro* by LGTV [151]. This shows that IRF-7 protects astrocytes from infection as well as viral replication. A higher propagation of LGTV in IRF-7$^{-/-}$ astrocytes also exposes more vulnerable neurons to the virus.

This change in tissue tropism indicates a protective function of IRF-7 especially in astrocytes.

3.9. The type I IFN response against LGTV in the brain is independent of IRF-7

For the maintenance of CNS functionality, microglia and astrocytes but also neurons themselves take active part in pathogen defence, independent from infiltrating immune cells [169][195][216]. This can be realized by secretion of growth factor or anti-inflammatory cytokines [202]. Cells produce for instance local type I IFN which is highly protective in viral infections [217]. IFN is secreted and acts in a paracrine and autocrine manner [218]. Thereby survival of the producer cells but also of the surrounding population is ensured [157].

Neurons were able to secrete type I IFN upon LGTV infection and this was independent of the presence of IRF-7. It was shown by others that only few neurons actually participate and proposed a restriction for IFN production in these cells [169].

Astrocytes were described as main IFN-β producers in viral infection of the olfactory bulb with vesicular stomatitis virus (VSV) [165]. Here, we show that infected IRF-7$^{-/-}$ astrocytes secreted very high amounts of type I IFN in LGTV infection *in vitro*. Interestingly, the ability of astrocytes to produce IFN was not impaired in the absence of IRF-7. This shows that infected astrocytes do not have to rely on the positive feedback loop mediated by IRF-7,

to induce a protective type I IFN responses. The upregulation of the response is merely regulated by the strength of the activation stimulus.
Similarly, microglia showed the ability to induce higher type I IFN responses in the absence of IRF-7.
The mechanism of this high, but IRF-7 independent IFN production is unclear. It was shown here, that in the CNS LGTV mediated IFN induction is at least partially due to an increase in IFN-β expression. IFN-α induction or compensatory mechanisms were not investigated further.
The IFN-β induction in the olfactory bulb was recently described as dependent on RIG-I signalling adaptor molecule IPS-1 [151]. However, the signal amplitude of IFN-β induction I in the absence of IRF-7 corresponded clearly to LGTV replication. Even though the olfactory bulb might be more vulnerable to LGTV infection, type I IFN induction in the absence of IRF-7 is not regionally restricted.
This raises questions about the positive feedback mechanism of IRF-7. The production of protective IFN by brain resident cells in the absence of important type I IFN regulators such as IRF-7 might sustain safeguarding mechanisms of the CNS. Many viruses are known to interfere with type I IFN components to evade immune recognition [173][219,220]. Specific targeting of IRF-7 by neurotropic viruses might be responsible for evolutionary pressure on the immune system to develop strategies that are IRF-7 independent [219][221]. Still, mechanisms to fine tune the antiviral response are probably impaired in the absence of IRF-7 [29]. Additionally, the absence of IRF-7 is associated with higher viral replication and decreased survival chances in many infections [54][71]. Therefore, this regulatory factor is not indispensable for the immune response of these animals.

3.10. Effect of type I IFN alterations on inflammatory processes in the CNS

Type I IFNs are employed as anti-inflammatory therapeutics in CNS diseases such as virus induced encephalitis or autoimmune diseases like multiple sclerosis (MS) [222,223]. Here it was shown that high amounts of IFN-β are induced in the absence of IRF-7. It is not clear, if this response is protective or if it has even detrimental effects on animal health. Whereas IFN exhibits anti-inflammatory effects in MS, we see enhanced inflammation in the absence of IRF-7 [223]. A possible explanation might be provided by the altered cell activation in the absence of IRF-7. As already mentioned above, reduced levels of IRF-7 can be associated with a switch to a M1 phenotype in macrophages and microglia [224]. A pro-inflammatory set of cytokines and chemokines is produced by these cells [225,226]. In contrast, anti-inflammatory effects of IFN in MS were due to alterations

in the T cell response [227,228]. Even though we could see increased infiltration of T cells, it is not clear if these cells contribute to viral clearance in LGTV and TBEV infection. The role of specific immune cell populations might therefore also influence the effectiveness of IFN treatment.

Even though we see increased inflammation in the absence of IRF-7 despite type I IFN production, we can not exclude a protective role for IFN induction in the CNS. It is quite possible that type I IFN still dampens the immune response in these animals. If type I IFN would be absent, there might be an even more severe inflammation. Excessive immune responses and a lack of type I IFN might be the cause of death in IFNAR$^{-/-}$ and IPS-1$^{-/-}$ animals [20][151].

It can here only be concluded that the production of type I IFN will modulate the immune response of IRF-7$^{-/-}$ animals and contributes to viral defence.

3.11. Implications for Flavivirus infection in humans

Even though IRF-7 is dispensable for survival of systemic LGTV infection the virus is able to persist in the brain. High induction of type I IFNs, an increased inflammatory response and immune cell activation in the absence of IRF-7 did not result in viral clearance in the acute phase of infection.

This has direct indications for patients infected with neurotropic viruses. Defects in the type I IFN response might not lead to death, but merely to more pronounced encephalitis or meningitis in humans. Inflammation related cytotoxic effects might results in neurological long-term effects that are often seen in patients that suffered from neurotropic infection [229–231]. Viral persistence might also be responsible for neurological sequelae, which was regularly observed in TBEV patients [88][229]. In this context, it is not clear if treatment with type I IFN is a sufficient therapeutic option for TBEV or other virus associated infections in the CNS [232]. This work clearly shows that more detailed knowledge of the immune response in the CNS is needed to efficiently fight neurotropic infections in humans.

4. Material and Methods

4.1. Materials

4.1.1. Consumables

All consumables used for this work are listed below (Table 1).

Tab.1: List of Consumables

Material	Detail	Manufacturer
Biosphere® Filter Tips	20 µl, 200 µl, 1000 µl	Sarstedt AG
Cell Star Cell Culture Dish	PS, 145 x20 mm, vent.	Greiner bio-one
Cell strainer	40 µm, 70 µm	BD Falcon
Ceramic spheres		MP Biomedicals
Cover glasses	Ø 13 mm	VWR
	24 x 50 mm	Carl Roth
Disposable Base molds	37x 24x 10 mm	Ted Pella Inc.
Embedding molds	13x 8x 5mm	Plano GmbH
Inject® single use syringe	2 ml, 5 ml, 10 ml, 20 ml	B Braun Medical Corp.
Micro Fine™ Insulin syringe	U-100, 0.5 ml, 0.33 mm x12.7 mm	Becton Dickinson GmbH
Microscope slides	Cut edges, frosted	VWR
	Menzel Gläser Superfrost®, cut edges	Thermo Fisher Scientific Inc.
Nunc® Micro Well™ plate	96 well, Polystyrene, round bottom	Thermo Fisher Scientific Inc.
Pasteur pipettes	150 mm, glass	Brand AG
Safe Seal Micro Tubes	1.5 ml, 2 ml	Sarstedt AG
Serological pipette	2 ml, 5 ml, 10 ml	Sarstedt AG
Single-use syringe	Omnican® F, 1 ml, 0.3 mm x 12 mm	B Braun Medical Corp.
Sterican® cannula	0.6 mm x 25 mm	B Braun Medical Corp.
	0.45 mm x 12 mm	
TC Dish 100	Std.	Sarstedt AG
TC Flask	T75, T25, Std, vent. Cap	Sarstedt AG
TC Plate	Std. F, 6 well, 12 well, 24 well, 48 well, 96 well	Sarstedt AG
Tip Stack Pack	20 µl, 200 µl, 1000 µl	Sarstedt AG
Tube	Concical bottom, 50 ml, 15 ml	Sarstedt AG

Tube	U-bottom bulk, 1.4 ml	Micronic
Tube	2 ml, colourless, sterile, screwtop	Biozym Scientific GmbH
Tube	5 ml, 75 x12 mm, PS	Sarstedt AG

4.1.2. Chemicals and reagents

Chemicals and reagents are depicted in Table 2.

Tab.2: Chemicals and reagents

Chemicals/Reagents	**Manufacturer**
Albumin Fraction V	Boehringer Mannheim GmbH
Avicel	FMC Corporation
Beetle Lysis Juice	PJK GmbH
Chloroform	Fluka Analytical
D-luciferin (*in vivo*)	SynChem OHG
D-Luciferin (*in vitro*)	PJK GmbH
Ethanol	J.T. Baker
Goat serum	Sigma Aldrich
Isoflurane	Baxter
Isopropanol	J.T. Baker
Methanol	J.T. Baker
Neo-Mount®	Merck KGoA
Percoll™	GE Healthcare
peqFECT	VWR PeqLab
peqGOLD TriFast	VWR PeqLab
Poly L-Lysin	Sigma Aldrich
Roti®-Histofix 4 %	Carl Roth GmbH
Succhrose	Merck KGoA
Tissue Tek O.C.T. compund	Sakura
Triton X-100	Serva Electrophoresis GmbH
Tween-20	Sigma Aldrich
Tween-80	Sigma Aldrich

4.1.3. Buffer and media

All media and supplements were obtained from Gibco® Life Technologies, InSCREENeX, Invitrogen, Merck KGoA, Sigma Aldrich and Biochrom AG. Specific buffers are described in the according section.

1x PBS
140 mM NaCl, 27 mM KCl, 7.2 mM Na_2HPO_4,14.7 mM KH_2PO_4,
pH 6.8-7

4.1.5. Virus

Langat virus strain TP21 (G. Dobler) was used for all experiments. The virus was propagated in Vero B4 cells and virus concentration determined by FFU Assay.

4.1.6. Laboratory Equipment

All devices used for this thesis and the according manufacturers are listed below (Table 3).

Tab.3: Laboratory equipment

Device	Type	Manufacturer
Autoclave	Dampf Sterilisator 6-6-6-HS1, FD	Belimed
Current Power Supply	Power Pac 1000	Bio-Rad Laboratories
Centrifuges	Biofuge Pico	Heraeus
	Biofuge Fresco	Heraeus
	CS-15R	Beckmann Coulter
	Cytospin 3	Shandon
Flow cytometer	LSR II	Becton Dickinson
Homogenisator	Fastprep 24	MP Biomedicals
Incubator	C200	Labotect
In vivo Imaging system	Xenogen IVIS System	Caliper
Luminometer	9507	Berthold Lumat LB
Magnetic stirrers hot plate	RET basic	IKA Werke GmbH
Micropipettes	10 µl, 20 µl, 100 µl, 200 µl, 1000 µl	Gilson
Microscopes	Confocal, LSM 510 Meta	Carl Zeiss
	Labovert	Leica

	Olympus CK2	Leica
MilliQ Unit	Biocel Millipore	Merck KGoA
Microwave	Pro 825	Whirlpool
Nitrogen Tank	17 K, HarscoK-Series	Tayloe Wharton
pH Meter	M340	Beckmann
Photometer	NanoDrop-1000	PeqLab
Pipette controller	Accu Jet pro	Brand GmbH
Scale	Precision weighting scale	Sartorius
Shaker	Duomax 1030	Heidolph
Sterile work bench	Herasafe	Heraeus
Thermomixer	Thermomixer compact	Eppendorf AG
Water bath	3044	ROWA

4.1.7. Computer software

Data collection, calculation and processing were done with Microsoft (MS) Office Professional 2010 Word, Excel and Power Point. To obtain microscopic pictures Automated interactive microscope (AIM) program and ZEN imaging software were used. Images were processed with ImageJ (National Institute of Health). *In vivo* images were processed with Living Image 3.2 software. Flow cytometry data were acquired with the LSR II program FACSDiva™ and FlowJo v10. Graphs and Statistical analysis were generated by GraphPad Prism.

4.2. Methods

4.2.1. Animal experiments

Ethics statement

The animal experiments were performed according to the guidelines of the German Animal welfare Law (TierSchG BGBl. S. 1105; 25.05.1998). The approved animal code for the experiments is 11/0528.

Animal housing

Animals were bred and cared for at the animal facility of the Helmholtz Centre for Infection research in Braunschweig or the animal house at the Otto-von-Guericke University, Magdeburg. C57BL/6 WT animals were obtained from ENVIGO. Transgenic mice on a

C57BL/6 background were kept under SPF conditions and the genotype was verified by PCR.
Mice were maintained in individually ventilated IVC Green Line cages (Tecniplast Germany GmbH) separated according to their gender. Mice had access to food and water ad libitum and were kept under standardized conditions, such as a 12 to 12 or 14 to 10 hour light-dark cycle, 50 lux illuminance, at 55 % humidity and 22 °C.

Anesthesia

Mice were anesthetized with 100 µl/ 10 g body weight of a 10 % ketamine (WDT), 5 % Xylazin (CD-Pharma) mixture in 0.9 % NaCl for i.c. injections.
For *in vivo* imaging animals were anesthetized continuously with Isoflurane (Baxter) in a rodent anesthesia chamber or the IVIS 200 integrated anesthesia unit.

Virus infection

6-12 week old mice were used for experiments. Animals were intraperitoneally injected with 100 µl 1x 10^4 FFU LGTV TP21. For intracranial infection mice were anesthetised as described above. Unconscious mice were injected intra cranially with 20 µl 1x 10^1 or 1x 10^2 FFU LGTV into the posterior fontanel. Virus was always diluted in 1x PBS.

***In vivo* Imaging**

IFN-$\beta^{+/\Delta luc}$, IFN-$\beta^{+/\Delta luc}$IRF-$3^{-/-}$ and IFN-$\beta^{+/\Delta luc}$IRF-$7^{-/-}$ mice were systemically infected with LGTV. Prior to *in vivo* imaging, mice were inoculated intravenously with 100 µl D-luciferin (150 mg/kg in PBS, SynChem OHG). Mice were anesthetized and monitored in the IVIS 200 imaging system (CaliperLS) for 2 minutes. The relative intensity of emitted light was detected and can be displayed as a pseudocolor image ranging from red (high) to blue (low). Signals were measured as photon flux and quantified as radiance p/sec/cm^3/sr. For image acquiring and processing the Living Image 3.2 software was used.

Blood isolation

Mice were sacrificed by CO_2 asphyxiation. The body cavity was opened and blood drawn with a single use syringe directly from the heart. To obtain serum, blood was allowed to coagulate for 2h at RT and was centrifuged down for 30 min at 2000 xg. The clear layer

was collected as serum and stored at -20 °C. For inactivation, serum was incubated for 20 min at 56 °C.

4.2.2. Cell culture

Dulbecco´s Modified Eagle Medium (DMEM), supplemented

DMEM (Gibco® Life Technologies) was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamine, 1 mM Natrium Pyruvate and 10 % fetal calf serum (FCS).

RPMI 1640, supplemented

RPMI 1640 (Gibco® Life Technologies) was supplemented with 10% FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1 mM 2-ME.

Gey´s balanced salt solution (GBSS), supplemented

0.22 g $CaCl_2$ x H_2O, 0.37 g KCL, 0.03 g KH_2PO_4, 0.21 g $MgCl_2$ x6 H_2O, 0.07 g $MgSO_4$ x7 H_2O, 8 g NaCl, 0.227 g $NaHCO_3$, 0.120 g Na_2HPO_4, 1 g D-Glucose were filled up to 1l H_2O, filtered and keep at 4°C.

Neurobasal Medium, supplemented

2 % B27 (Gibco® Life Technologies), 1x N-2 (Gibco® Life Technologies) and 0.5 mM Glutamine were added to Neurobasal medium (Gibco® Life Technologies).

Trypsin-EDTA

8 mM EDTA, 0.1 % trypsin, 1x PBS

Cells were handled only under a sterile workbench with vertical air flow. All media and buffer were autoclaved or filtered and pre-warmed to 37 °C before use. Working materials were disinfected with 70 % Ethanol prior to work. Cells were grown at 37 °C with 5 % CO_2 and 95 % relative humidity. Cells were passaged when they reached 90 % confluency. Cells were washed in 1x PBS and incubated with Trypsin-EDTA for 1 min at 37 °C. The enzymatic reaction was stopped with supplemented DMEM and cells were transferred into a fresh flask with new medium.

Immortalized kidney derived epithelial Vero B4 cells from green monkey were maintained in supplemented DMEM. Epithelial cells IEC-Mx2Luc-10 isolated from Mx2-Luc reporter mice were grown in IEC Medium (InSCREENeX GmbH) [233][234].

Primary hippocampal neurons

E18.5 old WT and IRF-7$^{-/-}$ mouse embryos were sacrificed by decapitation and brains kept in 4 °C supplemented GBSS. Hippocampi were isolated and enzymatically disintegrated in trypsin supplemented medium at 37 °C for 30 min. Cells were then separated by mechanical forces using sterile pasteur pipettes. Cells were counted and 70.000 primary hippocampal neurons seeded on Poly-L-Lysine coated sterile cover slips in supplemented Neurobasal medium. Cells were differentiated for three weeks with a partial medium exchange every 7 days and inocculated with LGTV at an MOI of 0.001 for 1.5 h. Cells were supplemented with their own media and incubated for 24, 48 and 72 hpi. Supernatants were harvested and tested for IFN secretion and quantified for viral particles.

Primary astrocytes and microglia

Postnatal mice at the age of P1-P3 were killed by decapitation and whole brains homogenized into 4 °C cold HBSS (Gibco®). Cell aggregates were disintegrated in DMEM supplemented with 10% FCS, 1% N-2, 0.5 mM Glutamine, 0.1 U/mL penicillin and 0.1ug/mL streptomycin. 2,5x 10^4 cells/ cm^2 were seeded on Poly D-Lysine coated (50 µg/ml) culture flasks and medium was changed 24 hours after isolation. Medium was renewed every 7-8 days and cells passaged at least three times before infection experiments. From these mixed glia cultures, microglia were removed by shaking at 600 rpm, 1 h, RT. 70.000 astrocytes or microglia were seeded on poly-D Lysine coated cover slips. Cells were infected with LGTV at an MOI 0.1 for 1 h or transfected by peqFECT with different concentrations of polyI:C. To determine secreted type I IFN and to quantify the virus, supernatants were harvested for infections at 24, 48 and 72 hpi and for polyI:C treatment, 24 hpt.

Bone marrow derived DCs and macrophages

Bone marrow were isolated by flushing femur and tibia of mice with 1x PBS. Cells were washed with 1x PBS and counted. Cells were seeded at a density of at least 1.5 x 10^6 cells/ ml in supplemented RMPI 1640 medium. The X63-GM-CSF cell line was employed for GM-CSF production (kindly provided by Dr. Brigitta Stockinger, Division of Molecular Immunology, National Institute of Medical Research, London, U.K.) and FL B16 cells for Flt-3 secretion [235].
BMDCs were cultivated for 8 days supplemented with 100ng/ml GM-CSF to generate adherent macrophages and mDCs suspension cultures. Similarly, 100 ng/ml Flt-3 was used to differentiate pDCs from BMDCs. The medium was renewed once, 4 days after seeding, by replacement of two-thirds of the medium. Differentiated macrophages, mDCs and pDCs were infected with LGTV at an MOI of 0.5 for 1h at 37°C. Supernatants were collected at 24, 48, 72 hpi and viral replication and type I IFN production were quantified. Cells were fixated and by cytospin centrifugation (800 rpm, 8 min, RT) transferred to microscope slides. Cells were stained by immunohistochemistry and at least 70 cells per sample (n=5) quantified for infected cells per DAPI positive cells.

4.2.3. Focus Forming Unit Assay

Infection medium

1x DMEM, supplemented with 2 % FCS, 2 mM Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Avicel-DMEM media

1.5% Avicel RC/CL and 2x DMEM were used in a 1:1 ratio and supplemented with 10 % FCS, 2 mM Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Permeabilization buffer

0.5 % Triton X-100 and 20mM glycine in 1x PBS.

Wash solution

All incubation steps were followed by three wash steps with 0.05 % Tween 80 in 1x PBS.

LGTV was quantified from supernatants by focus-forming assay as described previously [151]. Vero B4 monolayers were incubated with serial dilutions of LGTV samples in

infection medium for 1h at 37 °C. After removing the inoculum, cells were overlaid with Avicel-DMEM medium and after 24, 48 and 72h, cells were fixed with 6 % paraformaldehyde. All further steps were performed at RT. Cells were permeabilized for 20 min in permeabilization buffer and LGTV specific mouse anti-TBEV E-Protein MoAb 19/1786 [236] was used to detect viral foci. A secondary rabbit anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Jackson, # 315-035) was used for detection and LGTV positive foci were visualized by TrueBlue staining (KPL, Gaithersburg).

4.2.4. Flow Cytometry

FACS Buffer

2 % FCS in 1x PBS was used for washing and antibody dilution.

Systemically infected as well as uninfected mice were killed by CO_2 inhalation. Mice were perfused trans-cardially by 20 ml 1x PBS. Brains and spleens were harvested and organs homogenized through a 70 µm cell strainer into DMEM medium. A discontinuous 30 to 70 % percoll gradient was used to separate cells in brain samples. Total cells per brain or spleen were counted by a Neubauer chamber. All samples were saturated with FcR Blocking reagent (MiltenyiBiotec) and stained with antigen specific fluorescent antibodies (Table 4). Cells were fixed with 2 % Histofix diluted in 1x PBS and the analysis was done by BD LSRII using BD FACS Diva and FloJo software (FloJo V10). Single cell staining of spleen were used for manual compensation in all experiments.

Tab.4: Antibodies for flow cytometry analysis.

Antigen	Species	Reactivity	Fluorophor	Company
B220	Rat	Mouse	PE-Cy5	eBioscience
CD4	Rat	Mouse	APC	BD Pharmingen
CD8a	Rat	Mouse	Pacific Blue	BioLegend
CD11b	Rat	Mouse	PE-Cy7	eBioscience
CD11c	Armenian Hamster	Mouse	FITC	eBioscience
CD45	Rat	Mouse	PerCP-Cy5.5	eBioscience
CD80	Armenian Hamster	Mouse	PE	eBioscience
Ly-6C	Rat	Mouse	APC-Cy7	BioLegend
Ly-6G	Rat	Mouse	PE eFlour 610	eBioscience

Isolated cells from spleen were separated according to their surface marker expression as displayed in Figure 38.

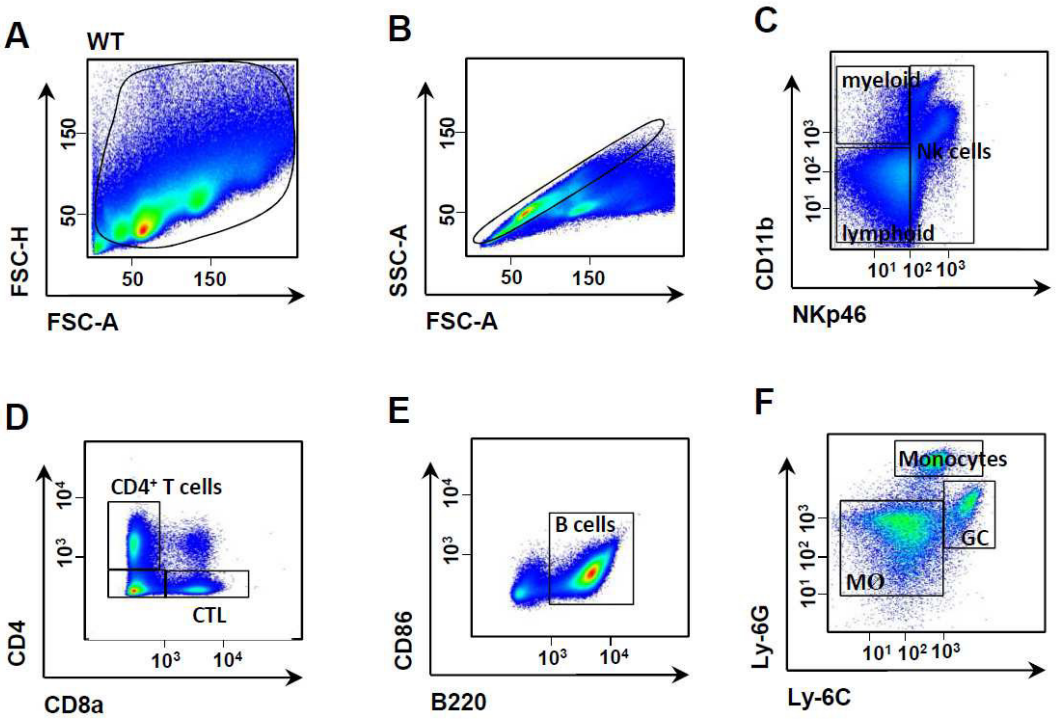

Fig.38: FACS Gating of splenocytes. Cells were isolated from infected and uninfected animals and splenocytes were measured by Flow Cytometry. Gating of infected WT cells is representatively described here. Dead cells and doublets were excluded by forward scatter (FSC-A and FSC-H) and sideward scatter (SSC-A) as illustrated (A, B). Single cells were separated by CD11b expression into $CD11b^+$ myeloid, $CD11b^-$ lymphoid and $Nkp46^+$ NK cells (C). Lymphoid cells (C) were characterized further by CD8a and CD4 expression into $CD4^+$ T cells and cytotoxic T cells (CTL) as shown (D). CD8a and CD4 negative lymphoid cells were identified by B220 expression as B cells (E). Myeloid cells (C) were segregated by Ly-6C and Ly-6G expression into macrophages, granulocytes (GC) and monocytes as depicted (F).

Isolated cells from brain were characterized as shown below (Fig. 39).

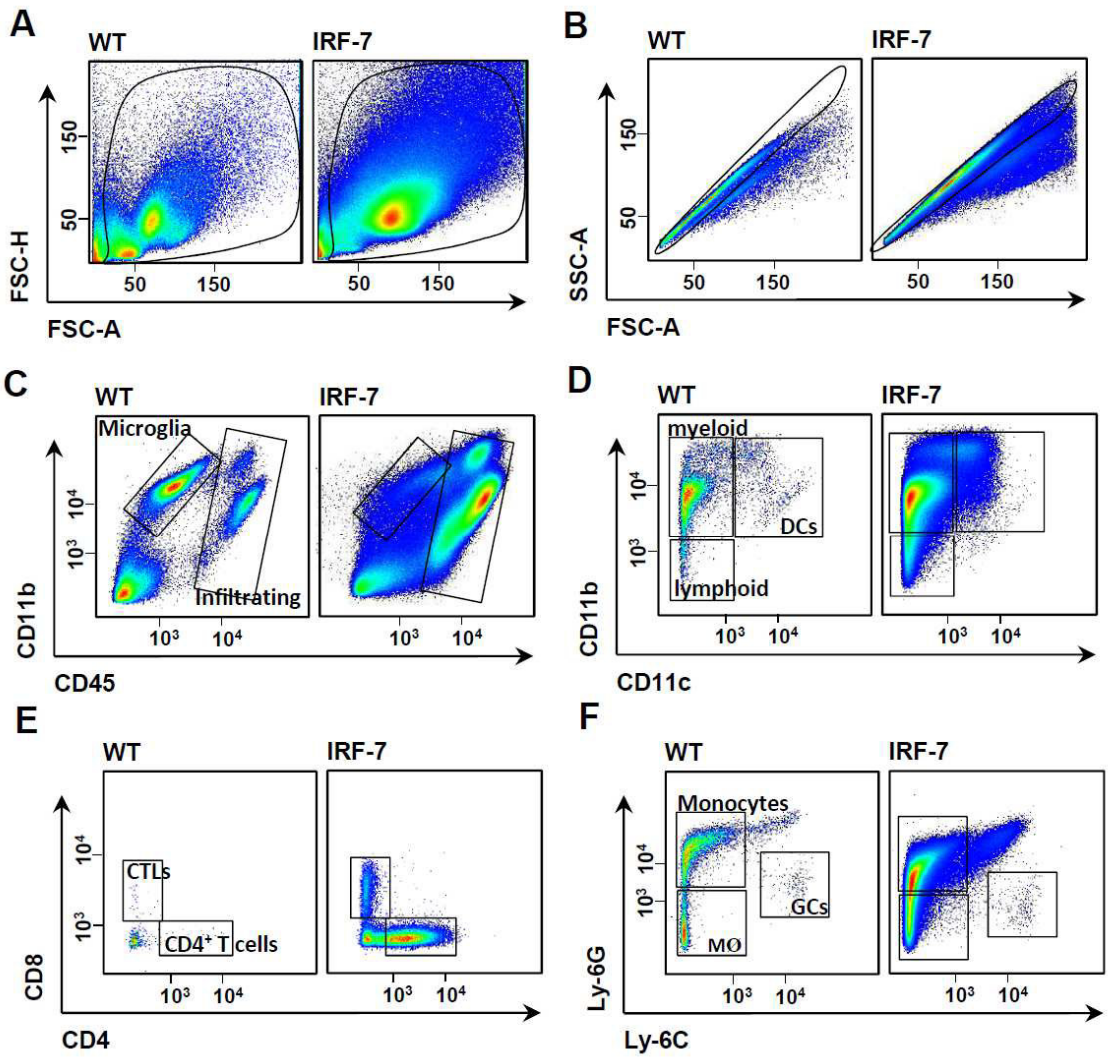

Fig.39: FACS Gating of brain isolates. Cells were isolated from brains by percoll gradient of infected and uninfected animals as described above. Flow Cytometry analysis of infected WT and IRF-$7^{-/-}$ are shown as representatives. Cell aggregates and doublets were excluded from the analysis by forward and sideward scatter (A, B). Infiltrating immune cells were segregated from brain resident microglia by CD45 expression (C). Infiltrating cells were further characterized by CD11b and CD11c expression into $CD11b^+$ myeloid cells, $CD11b^-$ lymphoid cells and $CD11c^+$ DCs as depicted (D). The lymphoid population contained $CD4^+$ and $CD8^+$ T cells (E). The myeloid population was separated again by Ly-6C and Ly-6G expression into macrophages, granulocytes and monocytes as illustrated (F).

4.2.5. Immunohistochemistry and Histology

Citrate buffer

10 mM $Na_3C_6H_5O_7$ x$2H_2O$ and 0.05 % Tween 20 were dissolved in MilliQ and adjusted to pH 6.0.

Cells from cell culture experiments were fixed with Histofix for 30 min at RT under the hood. Prior to staining with chicken anti-NS3 antibody, cells needed to undergo heat-induced antigen retrieval. Therefore these cells were submerged in citrate buffer and heated to 100 °C for 2 min.

For histology, mice were killed by CO_2 asphyxiation and perfused with 20 ml 1x PBS. 40 ml 4 % Histofix.

Fixed cells or tissues were then washed for two times in 1x PBS and incubated for 1h at RT in blocking solution containing 10 % goat serum, 0.2 % Triton X-100, 1 % Albumin in 1x PBS. The first antibody was added for 1h at RT or 4 °C o.n. in 0.2 % Triton, 10 % goat serum in 1x PBS. Cells were washed three times for 5 min in 1x PBS and then the second antibody was added for 1h at RT or 4 °C o.n.. Antibodies used for staining are shown in Table 5 and 6 below. Pictures were taken at the confocal microscope LSM 510 Meta at a magnification of 40x.

Tab. 5: Primary antibodies used for IHC and histology

Antigen	Species	Reactivity	Fluorophor	Clone	Company
B220	Rat	Mouse	PE	RA3-6B2	BD Pharmingen
CD8a	Rat	Mouse	PE	53-6.7	BD Pharmingen
CD11b	Rat	Mouse	PE	M1/70	eBioscience
E Protein	Mouse	Virus	none	MoAb 19/1786	[236]
F4/80	Rat	Mouse	PE	BM8	eBioscience
GFAP	Rabbit	Mouse	none	polyclonal	SYnaptic SYstems
GFAP	Guinea pig	Mouse	none	polyclonal	SYnaptic SYstems
IBA-1	Rabbit	Mouse	none	polyclonal	SYnaptic SYstems
NeuN	Guinea pig	Mouse	none	polyclonal	SYnaptic SYstems
NS3	chicken	Virus	none		

Tab.6: Secondary antibodies used for IHC and histology

Species	Reactivity	Fluorophor	Company
Goat	Mouse IgG $F(ab')_2$	Alexa Flour647	Jackson Laboratories Inc.
Goat	Guinea pig IgG (H+L)	Alexa Flour 488	Jackson Laboratories Inc.
Goat	Rabbit	Cy3	Dianova
Rabbit	Chicken IgY (H+L)	FITC	ThermoScientific

4.2.6. Interferon Reporter Assay

IFN-sensitive epithelial cells from Mx2-Luc reporter mice were grown in IEC medium [234]. Cells were overlaid with supernatants or heat inactivated serum samples as described previously [233]. Serial dilutions of IFN-β were used to determine a standard curve. The inoculum was removed after 24h at 37 °C and cells were lysed in Reporter Lysis buffer (Promega). Luciferase activity was determined by D-Luciferin conversion and Relative light units (RLU) measured by Berthold Luminometer Luma 9507.

4.2.7. IFN quantification from organs of IFN-$\beta^{+/\Delta luc}$ mice

IFN-$\beta^{+/\Delta luc}$, IFN-$\beta^{+/\Delta luc}$IRF-$3^{-/-}$ and IFN-$\beta^{+/\Delta luc}$IRF-$7^{-/-}$ mice were infected with 1x 10^4 FFU LGTV. Uninfected as well as infected animals were sacrificed after 0,2,4, and 7 dpi by CO_2 inhalation and perfused with 20 ml 1x PBS into the heart. Different organs and lymphoid tissues were isolated, frozen in liquid nitrogen and stored at -20 °C. For quantification of luciferase activity, organ samples were weighed and overlaid with Reporter Lysis Buffer (Promega). The tissue was homogenized by Fast Prep-24 and centrifuged at 2000 rpm for 45 min at 4 °C. Supernatants were collected and frozen at -20 °C. Thawed supernatants were added to D-Luciferin and luciferase activity measured by Berthold Luminometer. Results are depicted as RLU calculated to 1 ml and normalized to 1 g tissue weight. Due to the low weight of lymph nodes, these values were not normalized to tissue weight.

4.2.8. Gene expression analysis

TriFast was added to mouse organs and tissue homogenized by Fast Prep-24. RNA isolation was done as described [237]. RNA was extracted from serum by QIAamp Viral RNA Mini Kit (Cat.No. 52904, QIAGEN. RNA concentrations were quantified by NanoDrop and 1µg RNA was used for cDNA synthesis with RNA First-strand cDNA synthesis kit (GE, Healthcare). QPCR was performed with KAPA SYBR FAST Universal Master Mix (PeqLab, # 07-KK4600-02). The murine primers CCL2 (forward primer 3´-GCCCCACTCACCTGCTGCTA-5´, reverse primer 3´-TTTACGGGTCATCACATTCAA-5´) and TNF-α (forward pimer 3´- GAACTGGCAGAAGAGGCACT -5´, reverse primer 3´-AGGGTCTGGGCCATAGAACT-5´) were used for qPCR. A KAPA probe FAST qPCR kit based on a LGTV NS3 Taq-Man assay (forward primer 5´AACGGAGCCATAGCCAGTGA 3´, reverse primer 5´AACCCGTCCCGCCACTC 3´, probe FAM-AGAGACAGATCCCTGATGGMGB) was used to quantify viral concentrations. Samples

were measured by Light Cycler 480 II (Roche). The program included 40 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 30 s) and elongation (72 °C, 10 s). CP values for LGTV were normalized to housekeeping gene β-Actin, calculated by the ΔCT method and arbitrary units determined based on uninfected WT samples.

5. References

[1] S. Binder, A.M. Levitt, J.J. Sacks, J.M. Hughes, R.L. Berkelman, R.T. Bryan, et al., Emerging infectious diseases: public health issues for the 21st century., Science. 284 (1999) 1311–3. doi:10.1126/science.284.5418.1311.

[2] T. Ishikawa, A. Yamanaka, E. Konishi, A review of successful flavivirus vaccines and the problems with those flaviviruses for which vaccines are not yet available, Vaccine. 32 (2014) 1326–1337. doi:10.1016/j.vaccine.2014.01.040.

[3] J. Whitehorn, S. Yacoub, K.L. Anders, L.R. Macareo, M.C. Cassetti, V.C. Nguyen Van, et al., Dengue Therapeutics, Chemoprophylaxis, and Allied Tools: State of the Art and Future Directions, PLoS Negl. Trop. Dis. 8 (2014) e3025. doi:10.1371/journal.pntd.0003025.

[4] R.J. Ulevitch, Therapeutics targeting the innate immune system, Nat. Rev. Immunol. 4 (2004) 512–520. doi:10.1038/nri1396.

[5] C.A. Gogos, E. Drosou, H.P. Bassaris, A. Skoutelis, Pro- versus Anti-inflammatory Cytokine Profile in Patients with Severe Sepsis: A Marker for Prognosis and Future Therapeutic Options, J. Infect. Dis. 181 (2000) 176–180. doi:10.1086/315214.

[6] F. Baeke, T. Takiishi, H. Korf, C. Gysemans, C. Mathieu, Vitamin D: modulator of the immune system, Curr. Opin. Pharmacol. 10 (2010) 482–496. doi:10.1016/j.coph.2010.04.001.

[7] R. Medzhitov, C.A. Janeway, Innate Immunity: The Virtues of a Nonclonal System of Recognition, Cell. 91 (1997) 295–298. doi:10.1016/S0092-8674(00)80412-2.

[8] F. Geissmann, M.G. Manz, S. Jung, M.H. Sieweke, M. Merad, K. Ley, Development of monocytes, macrophages, and dendritic cells., Science. 327 (2010) 656–61. doi:10.1126/science.1178331.

[9] E. Vivier, D.H. Raulet, A. Moretta, M.A. Caligiuri, L. Zitvogel, L.L. Lanier, et al., Innate or adaptive immunity? The example of natural killer cells., Science. 331 (2011) 44–9. doi:10.1126/science.1198687.

[10] A. Mantovani, M.A. Cassatella, C. Costantini, S. Jaillon, Neutrophils in the activation and regulation of innate and adaptive immunity, Nat. Rev. Immunol. 11 (2011) 519–531. doi:10.1038/nri3024.

[11] A. Le Bon, D.F. Tough, Links between innate and adaptive immunity via type I interferon, Curr. Opin. Immunol. 14 (2002) 432–436. doi:10.1016/S0952-7915(02)00354-0.

[12] J. Hellwage, T. Meri, T. Heikkilä, A. Alitalo, J. Panelius, P. Lahdenne, et al., The complement regulator factor H binds to the surface protein OspE of Borrelia

burgdorferi., J. Biol. Chem. 276 (2001) 8427–35. doi:10.1074/jbc.M007994200.

[13] L.L. Lovelace, C.L. Cooper, J.M. Sodetz, L. Lebioda, Structure of human C8 protein provides mechanistic insight into membrane pore formation by complement., J. Biol. Chem. 286 (2011) 17585–92. doi:10.1074/jbc.M111.219766.

[14] S.R. Marder, D.E. Chenoweth, I.M. Goldstein, H.D. Perez, Chemotactic responses of human peripheral blood monocytes to the complement-derived peptides C5a and C5a des Arg., J. Immunol. 134 (1985) 3325–31. http://www.ncbi.nlm.nih.gov/pubmed/3884709 (accessed September 30, 2016).

[15] G. Trinchieri, Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity, Annu. Rev. Immunol. (1995). http://www.annualreviews.org/doi/abs/10.1146/annurev.iy.13.040195.001343 (accessed September 20, 2016).

[16] C. Langrish, B. McKenzie, N. Wilson, IL-12 and IL-23: master regulators of innate and adaptive immunity, Immunological. (2004). http://onlinelibrary.wiley.com/doi/10.1111/j.0105-2896.2004.00214.x/full (accessed September 20, 2016).

[17] X.S. Liu, Z.G. Zhang, R.L. Zhang, S.R. Gregg, L. Wang, T. Yier, et al., Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke, J. Neurosci. Res. 85 (2007) 2120–2125. doi:10.1002/jnr.21359.

[18] T. Ganz, Defensins: antimicrobial peptides of innate immunity, Nat. Rev. Immunol. 3 (2003) 710–720. doi:10.1038/nri1180.

[19] A. Chalifour, P. Jeannin, J.-F. Gauchat, A. Blaecke, M. Malissard, T. N'Guyen, et al., Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production., Blood. 104 (2004) 1778–83. doi:10.1182/blood-2003-08-2820.

[20] E. Weber, K. Finsterbusch, R. Lindquist, S. Nair, S. Lienenklaus, N.O. Gekara, et al., Type I interferon protects mice from fatal neurotropic infection with Langat virus by systemic and local anti-viral response., J. Virol. 88 (2014) 12202–12212. doi:10.1128/JVI.01215-14.

[21] D.M. Moncada, S.J. Kammanadiminti, K. Chadee, Mucin and Toll-like receptors in host defense against intestinal parasites, Trends Parasitol. 19 (2003) 305–311. doi:10.1016/S1471-4922(03)00122-3.

[22] M. Yoneyama, T. Fujita, RNA recognition and signal transduction by RIG-I-like receptors, Immunol. Rev. 227 (2009) 54–65. doi:10.1111/j.1600-065X.2008.00727.x.

[23] B.L. Fredericksen, B.C. Keller, J. Fornek, M.G. Katze, M. Gale, Establishment and

maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1., J. Virol. 82 (2008) 609–616. doi:10.1128/JVI.01305-07.

[24] H. Oshiumi, K. Sakai, M. Matsumoto, T. Seya, DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-β-inducing potential, Eur. J. Immunol. 40 (2010) 940–948. doi:10.1002/eji.200940203.

[25] G. Versteeg, A. García-Sastre, Viral tricks to grid-lock the type I interferon system, Curr. Opin. Microbiol. (2010). http://www.sciencedirect.com/science/article/pii/S1369527410000706 (accessed August 11, 2016).

[26] M. Servant, B. Tenoever, R. Lin, Review: overlapping and distinct mechanisms regulating IRF-3 and IRF-7 function, J. Interf. (2002). http://online.liebertpub.com/doi/abs/10.1089/107999002753452656 (accessed August 9, 2016).

[27] D. Levy, I. Marié, A. Prakash, Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity, Curr. Opin. Immunol. (2003). http://www.sciencedirect.com/science/article/pii/S0952791502000110 (accessed July 18, 2016).

[28] I. Marie, J.E. Durbin, D.E. Levy, W. Au, Y. Su, N. Raj, et al., Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7, EMBO J. 17 (1998) 6660–6669. doi:10.1093/emboj/17.22.6660.

[29] M. Sato, H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, et al., Distinct and Essential Roles of Transcription Factors IRF-3 and IRF-7 in Response to Viruses for IFN-α/β Gene Induction, Immunity. 13 (2000) 539–548. doi:10.1016/S1074-7613(00)00053-4.

[30] A. Kröger, M. Köster, K. Schroeder, H. Hauser, P.P. Mueller, Review: Activities of IRF-1, J. Interf. Cytokine Res. 22 (2002) 5–14. doi:10.1089/107999002753452610.

[31] H.M. Lazear, A. Lancaster, C. Wilkins, M.S. Suthar, A. Huang, S.C. Vick, et al., IRF-3, IRF-5, and IRF-7 Coordinately Regulate the Type I IFN Response in Myeloid Dendritic Cells Downstream of MAVS Signaling, PLoS Pathog. 9 (2013) e1003118. doi:10.1371/journal.ppat.1003118.

[32] S.S. Ousman, J. Wang, I.L. Campbell, Differential regulation of interferon regulatory factor (IRF)-7 and IRF-9 gene expression in the central nervous system during viral infection., J Virol. 79 (2005) 7514–27. doi:10.1128/JVI.79.12.7514-7527.2005.

[33] M. Costa-Mattioli, N. Sonenberg, RAPping production of type I interferon in pDCs through mTOR, Nat. Immunol. (2008).

http://www.nature.com/ni/journal/v9/n10/abs/ni1008-1097.html (accessed August 15, 2016).

[34] T. Kawai, S. Sato, K.J. Ishii, C. Coban, H. Hemmi, M. Yamamoto, et al., Interferon-α induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6, Nat. Immunol. 5 (2004) 1061–1068. doi:10.1038/ni1118.

[35] K. Honda, H. Yanai, T. Mizutani, H. Negishi, N. Shimada, N. Suzuki, et al., Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling, Proc. Natl. Acad. Sci. 101 (2004) 15416–15421. doi:10.1073/pnas.0406933101.

[36] T. Kawai, S. Akira, Signaling to NF-κB by Toll-like receptors, Trends Mol. Med. 13 (2007) 460–469. doi:10.1016/j.molmed.2007.09.002.

[37] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol. (2010). http://www.nature.com/ni/journal/v11/n5/abs/ni.1863.html (accessed August 15, 2016).

[38] Y. Sun, M. Han, C. Kim, J. Calvert, D. Yoo, Interplay between interferon-mediated innate immunity and porcine reproductive and respiratory syndrome virus, Viruses. (2012). http://www.mdpi.com/1999-4915/4/4/424/htm (accessed August 15, 2016).

[39] C. Asselin-Paturel, G. Trinchieri, Production of type I interferons, J. Exp. Med. 202 (2005) 461–465. doi:10.1084/jem.20051395.

[40] S.S. Ousman, J. Wang, I.L. Campbell, Differential Regulation of Interferon Regulatory Factor (IRF)-7 and IRF-9 Gene Expression in the Central Nervous System during Viral Infection, J. Virol. 79 (2005) 7514–7527. doi:10.1128/JVI.79.12.7514-7527.2005.

[41] G. Tossing, New developments in interferon therapy., Eur. J. Med. Res. 6 (2001) 47–65. http://www.ncbi.nlm.nih.gov/pubmed/11313191 (accessed August 4, 2016).

[42] G. Jego, A.K. Palucka, J.-P. Blanck, C. Chalouni, V. Pascual, J. Banchereau, Plasmacytoid Dendritic Cells Induce Plasma Cell Differentiation through Type I Interferon and Interleukin 6, Immunity. 19 (2003) 225–234. doi:10.1016/S1074-7613(03)00208-5.

[43] L.C. Platanias, Mechanisms of type-I- and type-II-interferon-mediated signalling, Nat. Rev. Immunol. 5 (2005) 375–386. doi:10.1038/nri1604.

[44] A.N. Theofilopoulos, R. Baccala, B. Beutler, D.H. Kono, TYPE I INTERFERONS (α/β) IN IMMUNITY AND AUTOIMMUNITY, http://dx.doi.org/10.1146/annurev.immunol.23.021704.115843. (2004).

[45] C. Bogdan, The function of type I interferons in antimicrobial immunity, Curr. Opin. Immunol. 12 (2000) 419–424. doi:10.1016/S0952-7915(00)00111-4.

[46] A. Nakajima, Y. Sokawa, Induction of Blood 2′,5′-Oligoadenylate Synthetase Activity in Mice by Gastric Administration of Ovine IFN-τ, Http://www.liebertpub.com/jir. (2004).

[47] C.E. Samuel, Antiviral Actions of Interferons, Clin. Microbiol. Rev. 14 (2001) 778–809. doi:10.1128/CMR.14.4.778-809.2001.

[48] H. Arase, N. Arase, T. Saito, Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking, J. Exp. Med. 183 (1996) 2391–2396. doi:10.1084/jem.183.5.2391.

[49] G.J. Bancroft, R.D. Schreiber, G.C. Bosma, M.J. Bosma, E.R. Unanue, A T cell-independent mechanism of macrophage activation by interferon-gamma., J. Immunol. 139 (1987) 1104–7. http://www.ncbi.nlm.nih.gov/pubmed/3112223 (accessed August 4, 2016).

[50] R.P. Donnelly, S. V Kotenko, Interferon-lambda: a new addition to an old family., J. Interferon Cytokine Res. 30 (2010) 555–64. doi:10.1089/jir.2010.0078.

[51] H.-H. Hoffmann, W.M. Schneider, C.M. Rice, Interferons and viruses: an evolutionary arms race of molecular interactions, Trends Immunol. 36 (2015) 124–138. doi:10.1016/j.it.2015.01.004.

[52] L.B. Ivashkiv, L.T. Donlin, Regulation of type I interferon responses, Nat. Rev. Immunol. 14 (2013) 36–49. doi:10.1038/nri3581.

[53] S. Daffis, M.A. Samuel, M.S. Suthar, B.C. Keller, M. Gale, M.S. Diamond, Interferon Regulatory Factor IRF-7 Induces the Antiviral Alpha Interferon Response and Protects against Lethal West Nile Virus Infection, J. Virol. 82 (2008) 8465–8475. doi:10.1128/JVI.00918-08.

[54] S. Daffis, M.A. Samuel, M.S. Suthar, B.C. Keller, M. Gale, M.S. Diamond, Interferon Regulatory Factor IRF-7 Induces the Antiviral Alpha Interferon Response and Protects against Lethal West Nile Virus Infection, J. Virol. 82 (2008) 8465–8475. doi:10.1128/JVI.00918-08.

[55] K. Ozato, P. Tailor, T. Kubota, The Interferon Regulatory Factor Family in Host Defense: Mechanism of Action, J. Biol. Chem. 282 (2007) 20065–20069. doi:10.1074/jbc.R700003200.

[56] A. Legrand, N. Vadrot, B. Lardeux, A.F. Bringuier, R. Guillot, G. Feldmann, Study of the effects of interferon a on several human hepatoma cell lines: analysis of the signalling pathway of the cytokine and of its effects on apoptosis and cell proliferation, Liver Int. 24 (2004) 149–160. doi:10.1111/j.1478-3231.2004.00899.x.

[57] F. Lancillotti, V. Giandomenico, E. Affabris, G. Fiorucci, G. Romeo, G.B. Rossi, Interferon a-2b and Retinole Acid Combined Treatment Affects Proliferation and Gene Expression of Human Cervical Carcinoma Cells1, CANCER Res. 55 (1995)

3158–3164.

[58] K.S.A. Khabar, L. al-Haj, F. al-Zoghaibi, M. Marie, M. Dhalla, S.J. Polyak, et al., Expressed Gene Clusters Associated with Cellular Sensitivity and Resistance Towards Anti-viral and Anti-proliferative Actions of Interferon, J. Mol. Biol. 342 (2004) 833–846. doi:10.1016/j.jmb.2004.07.065.

[59] M. Miyamoto, T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, et al., Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements, Cell. 54 (1988) 903–913. doi:10.1016/S0092-8674(88)91307-4.

[60] M. Lohoff, G.S. Duncan, D. Ferrick, H.-W. Mittrücker, S. Bischof, S. Prechtl, et al., Deficiency in the Transcription Factor Interferon Regulatory Factor (Irf)-2 Leads to Severely Compromised Development of Natural Killer and T Helper Type 1 Cells, J. Exp. Med. 192 (2000) 325–336. doi:10.1084/jem.192.3.325.

[61] S. Marecki, M.J. Fenton, Review: The Role of IRF-4 in Transcriptional Regulation, J. Interf. Cytokine Res. 22 (2002) 121–133. doi:10.1089/107999002753452737.

[62] A.B. Pernis, Review: The Role of IRF-4 in B and T Cell Activation and Differentiation, J. Interf. Cytokine Res. 22 (2002) 111–120. doi:10.1089/107999002753452728.

[63] S. Marecki, M.L. Atchison, M.J. Fenton, Differential expression and distinct functions of IFN regulatory factor 4 and IFN consensus sequence binding protein in macrophages., J. Immunol. 163 (1999) 2713–22. http://www.ncbi.nlm.nih.gov/pubmed/10453013 (accessed August 8, 2016).

[64] T. Tamura, K. Ozato, Review: ICSBP/IRF-8: Its Regulatory Roles in the Development of Myeloid Cells, J. Interf. Cytokine Res. 22 (2002) 145–152. doi:10.1089/107999002753452755.

[65] H. Tsujimura, T. Tamura, H.J. Kong, A. Nishiyama, K.J. Ishii, D.M. Klinman, et al., Toll-Like Receptor 9 Signaling Activates NF- B through IFN Regulatory Factor-8/IFN Consensus Sequence Binding Protein in Dendritic Cells, J. Immunol. 172 (2004) 6820–6827. doi:10.4049/jimmunol.172.11.6820.

[66] T. Kondo, T. Kawai, S. Akira, C.A. Janeway, R. Medzhitov, S. Akira, et al., Dissecting negative regulation of Toll-like receptor signaling., Trends Immunol. 33 (2012) 449–58. doi:10.1016/j.it.2012.05.002.

[67] A.M. Delgado-Vega, M.E. Alarcón-Riquelme, S. V Kozyrev, S. Pestka, C. Krause, M. Walter, et al., Genetic associations in type I interferon related pathways with autoimmunity, Arthritis Res. Ther. 12 (2010) S2. doi:10.1186/ar2883.

[68] B.J. Barnes, P.A. Moore, P.M. Pitha, Virus-specific Activation of a Novel Interferon Regulatory Factor, IRF-5, Results in the Induction of Distinct Interferon Genes, J.

Biol. Chem. 276 (2001) 23382–23390. doi:10.1074/jbc.M101216200.

[69] M. Sato, H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction, Immunity. (2000). http://www.sciencedirect.com/science/article/pii/S1074761300000534 (accessed July 18, 2016).

[70] I. Marie, J.E. Durbin, D.E. Levy, W. Au, Y. Su, N. Raj, et al., Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7, EMBO J. 17 (1998) 6660–6669. doi:10.1093/emboj/17.22.6660.

[71] K. Honda, H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, et al., IRF-7 is the master regulator of type-I interferon-dependent immune responses., Nature. 434 (2005) 772–7. doi:10.1038/nature03464.

[72] L. Zhang, J. Zhang, Q. Lambert, C.J. Der, L. Del Valle, J. Miklossy, et al., Interferon Regulatory Factor 7 Is Associated with Epstein-Barr Virus-Transformed Central Nervous System Lymphoma and Has Oncogenic Properties, J. Virol. 78 (2004) 12987–12995. doi:10.1128/JVI.78.23.12987-12995.2004.

[73] D.E. Levy, I. Marié, E. Smith, A. Prakash, Review: Enhancement and Diversification of IFN Induction by IRF-7-Mediated Positive Feedback, J. Interf. Cytokine Res. 22 (2002) 87–93. doi:10.1089/107999002753452692.

[74] R. Lu, P.M. Pitha, Monocyte Differentiation to Macrophage Requires Interferon Regulatory Factor 7, J. Biol. Chem. 276 (2001) 45491–45496. doi:10.1074/jbc.C100421200.

[75] M.J. Ciancanelli, S.X.L. Huang, P. Luthra, H. Garner, Y. Itan, S. Volpi, et al., Infectious disease. Life-threatening influenza and impaired interferon amplification in human IRF7 deficiency., Science. 348 (2015) 448–53. doi:10.1126/science.aaa1578.

[76] K. Werme, M. Wigerius, M. Johansson, Tick-borne encephalitis virus NS5 associates with membrane protein scribble and impairs interferon-stimulated JAK-STAT signalling., Cell. Microbiol. 10 (2008) 696–712. doi:10.1111/j.1462-5822.2007.01076.x.

[77] G.S. Campos, A.C. Bandeira, S.I. Sardi, Zika Virus Outbreak, Bahia, Brazil., Emerg. Infect. Dis. 21 (2015) 1885–6. doi:10.3201/eid2110.150847.

[78] K.L. Mansfield, N. Johnson, L.P. Phipps, J.R. Stephenson, A.R. Fooks, T. Solomon, Tick-borne encephalitis virus - a review of an emerging zoonosis, J. Gen. Virol. 90 (2009) 1781–1794. doi:10.1099/vir.0.011437-0.

[79] V.-M. Cao-Lormeau, D. Musso, I. Leparc-Goffart, A. Nougairede, S. Cassadou, C. Prat, et al., Emerging arboviruses in the Pacific, Lancet. 384 (2014) 1571–1572.

doi:10.1016/S0140-6736(14)61977-2.

[80] J.S. Mackenzie, D.J. Gubler, L.R. Petersen, Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses, Nat. Med. 10 (2004) S98–S109. doi:10.1038/nm1144.

[81] F.X. Heinz, K. Stiasny, H. Holzmann, M. Grgic-Vitek, B. Kriz, A. Essl, et al., Vaccination and Tick-borne Encephalitis, Central Europe, Emerg. Infect. Dis. 19 (2013) 69–76. doi:10.3201/eid1901.120458.

[82] E.C. for D.P. and C. (ECDC)-H.C.U.-E. editorial team, Case report: Tick-borne encephalitis in two Dutch travellers returning from Austria, Netherlands, July and August 2011, (2011). http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20003 (accessed May 10, 2016).

[83] F.X. Heinz, C. Kunz, Tick-borne encephalitis and the impact of vaccination, in: Emerg. Control Zoonotic Viral Enceph., Springer Vienna, Vienna, 2004: pp. 201–205. doi:10.1007/978-3-7091-0572-6_18.

[84] E. Mohareb, I. Christova, A. Soliman, R. Younan, T. Kantardjiev, Tick-borne encephalitis in Bulgaria, 2009 to 2012, Eurosurveillance. 18 (2013) 20635. doi:10.2807/1560-7917.ES2013.18.46.20635.

[85] J. Suss, Tick-borne encephalitis in Europe and beyond--the epidemiological situation as of 2007., Euro Surveill. Bull. Eur. Sur Les Mal. Transm. = Eur. Commun. Dis. Bull. 13 (2008) 717–727. http://europepmc.org/abstract/med/18761916 (accessed May 10, 2016).

[86] Robert Koch Institut, FSME: Risikogebiete in Deutschland (Stand: Mai 2013), Epidemiol. Bull. (2013) p.151–166. https://www.google.de/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=0ahUKEwiHtsL-x6LOAhUIIsAKHWKEBM4QFggtMAI&url=https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2013/Ausgaben/18_13.pdf?__blob=publicationFile&us.

[87] European Commission, Amending Decision 2000/96/EC as regards tick-borne encephalitis and the category of vector-borne communicable diseases, OJ L 239/3; 5.9.2012. (2012). http://ec.europa.eu/health/communicable_diseases/early_warning/comm_legislation_en.htm.

[88] T.. Gritsun, V.. Lashkevich, E.. Gould, Tick-borne encephalitis, Antiviral Res. 57 (2003) 129–146. doi:10.1016/S0166-3542(02)00206-1.

[89] N. Hudopisk, M. Korva, E. Janet, M. Simetinger, M. Grgič-Vitek, J. Gubenšek, et al., Tick-borne Encephalitis Associated with Consumption of Raw Goat Milk, Slovenia,

2012, Emerg. Infect. Dis. 19 (2013). doi:10.3201/eid1905.121442.

[90] A.N. Shapoval, [Chronic forms of tick-borne encephalitis]., Zh. Nevropatol. Psikhiatr. Im. S. S. Korsakova. 74 (1974) 205–9. http://www.ncbi.nlm.nih.gov/pubmed/4282811 (accessed September 30, 2016).

[91] E.C. for D.P. and C. (ECDC)-H.C.U.-E. editorial team, Tick-borne encephalitis increasing in Sweden, 2011, (n.d.). http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19981 (accessed May 10, 2016).

[92] A. Loew-Baselli, E.-M. Poellabauer, B.G. Pavlova, S. Fritsch, M. Koska, R. Bobrovsky, et al., Seropersistence of tick-borne encephalitis antibodies, safety and booster response to FSME-IMMUN ® 0.5 ml in adults aged 18-67 years, Hum. Vaccin. 5 (2009) 551–556. doi:10.4161/hv.5.8.8571.

[93] C. Wittermann, A. Izu, E. Petri, D. Gniel, E. Fragapane, Five year follow-up after primary vaccination against tick-borne encephalitis in children, Vaccine. 33 (2015) 1824–1829. doi:10.1016/j.vaccine.2015.02.038.

[94] C. Kunz, TBE vaccination and the Austrian experience, Vaccine. 21 (2003) S50–S55. doi:10.1016/S0264-410X(02)00813-7.

[95] G. Kuno, G.J. Chang, K.R. Tsuchiya, N. Karabatsos, C.B. Cropp, Phylogeny of the genus Flavivirus., J. Virol. 72 (1998) 73–83. http://www.ncbi.nlm.nih.gov/pubmed/9420202 (accessed August 2, 2016).

[96] G.N. Leonova, S.I. Belikov, I.G. Kondratov, I. Takashima, Comprehensive assessment of the genetics and virulence of tick-borne encephalitis virus strains isolated from patients with inapparent and clinical forms of the infection in the Russian Far East, Virology. 443 (2013) 89–98. doi:10.1016/j.virol.2013.04.029.

[97] J.S. Porterfield, The basis of arbovirus classification., Med. Biol. 53 (1975) 400–5. http://www.ncbi.nlm.nih.gov/pubmed/1207193 (accessed September 30, 2016).

[98] G. Grard, G. Moureau, R.N. Charrel, J.-J. Lemasson, J.-P. Gonzalez, P. Gallian, et al., Genetic characterization of tick-borne flaviviruses: New insights into evolution, pathogenetic determinants and taxonomy, Virology. 361 (2007) 80–92. doi:10.1016/j.virol.2006.09.015.

[99] T. Chambers, C. Hahn, R. Galler, Flavivirus genome organization, expression, and replication, Annu. Rev. (1990). http://www.annualreviews.org/doi/pdf/10.1146/annurev.mi.44.100190.003245 (accessed September 30, 2016).

[100] V. Bakhvalova, V. Rar, S. Tkachev, V. Matveev, Tick-borne encephalitis virus strains of Western Siberia, Virus Res. (2000). http://www.sciencedirect.com/science/article/pii/S016817020000174X (accessed

August 2, 2016).

[101] A.G. Pletnev, V.F. Yamshchikov, V.M. Blinov, Nucleotide sequence of the genome and complete amino acid sequence of the polyprotein of tick-borne encephalitis virus, Virology. 174 (1990) 250–263. doi:10.1016/0042-6822(90)90073-Z.

[102] M.S. Marin, P.M. De A. Zanotto, T.S. Gritsun, E.A. Gould, Phylogeny of TYU, SRE, and CFA Virus: Different Evolutionary Rates in the Genus Flavivirus, Virology. 206 (1995) 1133–1139. doi:10.1006/viro.1995.1038.

[103] M. Haglund, S. Vene, M. Forsgren, G. Günther, B. Johansson, M. Niedrig, et al., Characterisation of human tick-borne encephalitis virus from Sweden, J. Med. Virol. 71 (2003) 610–621. doi:10.1002/jmv.10497.

[104] C.W. Mandl, L. Iacono-Connors, G. Wallner, H. Holzmann, C. Kunz, F.X. Heinz, Sequence of the genes encoding the structural proteins of the low-virulence tick-borne flaviviruses Langat TP21 and Yelantsev, Virology. 185 (1991) 891–895. doi:10.1016/0042-6822(91)90567-U.

[105] M.S. Campbell, A.G. Pletnev, Infectious cDNA Clones of Langat Tick-Borne Flavivirus That Differ from Their Parent in Peripheral Neurovirulence, Virology. 269 (2000) 225–237. doi:10.1006/viro.2000.0220.

[106] W.H. Price, I.S. Thind, R.D. Teasdall, W. O'Leary, Vaccination of human volunteers against Russian spring-summer (RSS) virus complex with attenuated Langat E5 virus., Bull. World Health Organ. 42 (1970) 89–94. http://www.ncbi.nlm.nih.gov/pubmed/5309521 (accessed August 9, 2016).

[107] A.G. Pletnev, G.G. Karganova, T.I. Dzhivanyan, V.A. Lashkevich, M. Bray, Chimeric Langat/Dengue Viruses Protect Mice from Heterologous Challenge with the Highly Virulent Strains of Tick-Borne Encephalitis Virus, Virology. 274 (2000) 26–31. doi:10.1006/viro.2000.0426.

[108] GN Leonova, Tick-Borne Encephalitis in Primorski Region., Virusol. Ecol. Asp. Vladivostok, Daln. . 189 (1997). https://scholar.google.de/scholar?q=Tick-Borne+Encephalitis+in+Primorski+Region.+Dalnauka&btnG=&hl=de&as_sdt=0%2C5.

[109] C.W. Mandl, F.X. Heinz, H. Holzmann, C. Kunz, M. Ecker, Infectious cDNA clones of tick-borne encephalitis virus European subtype prototypic strain Neudoerfl and high virulence strain Hypr., J. Gen. Virol. 78 (1997) 1049–1057. doi:10.1099/0022-1317-78-5-1049.

[110] N.L. Teterina, O.A. Maximova, H. Kenney, G. Liu, A.G. Pletnev, MicroRNA-based control of tick-borne flavivirus neuropathogenesis: Challenges and perspectives, Antiviral Res. 127 (2016) 57–67. doi:10.1016/j.antiviral.2016.01.003.

[111] A.A. Rumyantsev, B.R. Murphy, A.G. Pletnev, A Tick-Borne Langat Virus Mutant

That Is Temperature Sensitive and Host Range Restricted in Neuroblastoma Cells and Lacks Neuroinvasiveness for Immunodeficient Mice, J. Virol. 80 (2006) 1427–1439. doi:10.1128/JVI.80.3.1427-1439.2006.

[112] L. Mlera, J. Lam, D.K. Offerdahl, C. Martens, D. Sturdevant, C. V. Turner, et al., Transcriptome Analysis Reveals a Signature Profile for Tick-Borne Flavivirus Persistence in HEK 293T Cells, MBio. 7 (2016) e00314–16. doi:10.1128/mBio.00314-16.

[113] E.K. Wagner, E.K. Wagner, Basic virology, Blackwell Pub, 2008.

[114] E. Tonteri, P. Jokelainen, J. Matala, J. Pusenius, O. Vapalahti, Serological evidence of tick-borne encephalitis virus infection in moose and deer in Finland: sentinels for virus circulation., Parasit. Vectors. 9 (2016) 54. doi:10.1186/s13071-016-1335-6.

[115] M. Halevy, Y. Akov, D. Ben-Nathan, D. Kobiler, B. Lachmi, S. Lustig, Loss of active neuroinvasiveness in attenuated strains of West Nile virus: pathogenicity in immunocompetent and SCID mice, Arch. Virol. 137 (1994) 355–370. doi:10.1007/BF01309481.

[116] M.S. Smith, G.L. Bentz, J.S. Alexander, A.D. Yurochko, Human Cytomegalovirus Induces Monocyte Differentiation and Migration as a Strategy for Dissemination and Persistence, J. Virol. 78 (2004) 4444–4453. doi:10.1128/JVI.78.9.4444-4453.2004.

[117] M. Bray, T.W. Geisbert, Ebola virus: The role of macrophages and dendritic cells in the pathogenesis of Ebola hemorrhagic fever, Int. J. Biochem. Cell Biol. 37 (2005) 1560–1566. doi:10.1016/j.biocel.2005.02.018.

[118] M. LABUDA, J.M. AUSTYN, E. ZUFFOVA, O. KOZUCH, N. FUCHSBERGER, J. LYSY, et al., Importance of Localized Skin Infection in Tick-Borne Encephalitis Virus Transmission, Virology. 219 (1996) 357–366. doi:10.1006/viro.1996.0261.

[119] M. Hirano, K. Yoshii, M. Sakai, R. Hasebe, O. Ichii, H. Kariwa, Tick-borne flaviviruses alter membrane structure and replicate in dendrites of primary mouse neuronal cultures, J. Gen. Virol. 95 (2014) 849–861. doi:10.1099/vir.0.061432-0.

[120] E. Gelpi, M. Preusser, U. Laggner, F. Garzuly, H. Holzmann, F.X. Heinz, et al., Inflammatory response in human tick-borne encephalitis: analysis of postmortem brain tissue, J. Neurovirol. (2009).

[121] T.P. Monath, C.B. Cropp, A.K. Harrison, Mode of entry of a neurotropic arbovirus into the central nervous system. Reinvestigation of an old controversy., Lab. Invest. 48 (1983) 399–410.

[122] T. Wang, T. Town, L. Alexopoulou, J.F. Anderson, E. Fikrig, R. a Flavell, Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis., Nat. Med. 10 (2004) 1366–1373. doi:10.1038/nm1140.

[123] J. Dai, P. Wang, F. Bai, T. Town, E. Fikrig, Icam-1 participates in the entry of west nile virus into the central nervous system., J. Virol. 82 (2008) 4164–8. doi:10.1128/JVI.02621-07.

[124] M. a Samuel, H. Wang, V. Siddharthan, J.D. Morrey, M.S. Diamond, Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis., Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 17140–17145. doi:10.1073/pnas.0705837104.

[125] D.G. Baker, T.A. Woods, N.B. Butchi, T.M. Morgan, R.T. Taylor, P. Sunyakumthorn, et al., Toll-like receptor 7 suppresses virus replication in neurons but does not affect viral pathogenesis in a mouse model of Langat virus infection, J. Gen. Virol. 94 (2013) 336–347. doi:10.1099/vir.0.043984-0.

[126] E. Kindberg, S. Vene, A. Mickiene, Å. Lundkvist, L. Lindquist, L. Svensson, A functional Toll-like receptor 3 gene (TLR3) may be a risk factor for tick-borne encephalitis virus (TBEV) infection., J. Infect. Dis. 203 (2011) 523–8. doi:10.1093/infdis/jiq082.

[127] D. Hayasaka, N. Nagata, Y. Fujii, H. Hasegawa, Mortality following peripheral infection with tick-borne encephalitis virus results from a combination of central nervous system pathology, systemic, Virology. (2009). http://www.sciencedirect.com/science/article/pii/S0042682209002864 (accessed August 11, 2016).

[128] D. Michlmayr, S. V. Bardina, C.A. Rodriguez, A.G. Pletnev, J.K. Lim, Dual Function of Ccr5 during Langat Virus Encephalitis: Reduction in Neutrophil-Mediated Central Nervous System Inflammation and Increase in T Cell-Mediated Viral Clearance, J. Immunol. 196 (2016) 4622–4631. doi:10.4049/jimmunol.1502452.

[129] E. Kindberg, A. Mickiene, C. Ax, B. Akerlind, S. Vene, L. Lindquist, et al., A deletion in the chemokine receptor 5 (CCR5) gene is associated with tickborne encephalitis., J. Infect. Dis. 197 (2008) 266–9. doi:10.1086/524709.

[130] G.G. Prikhod'ko, E.A. Prikhod'ko, J.I. Cohen, A.G. Pletnev, Infection with Langat Flavivirus or Expression of the Envelope Protein Induces Apoptotic Cell Death, Virology. 286 (2001) 328–335. doi:10.1006/viro.2001.0980.

[131] G.G. Prikhod'ko, E.A. Prikhod'ko, J.I. Cohen, A.G. Pletnev, Infection with Langat Flavivirus or Expression of the Envelope Protein Induces Apoptotic Cell Death, Virology. 286 (2001) 328–335. doi:10.1006/viro.2001.0980.

[132] A.K. Overby, V.L. Popov, M. Niedrig, F. Weber, Tick-borne encephalitis virus delays interferon induction and hides its double-stranded RNA in intracellular membrane vesicles., J. Virol. 84 (2010) 8470–83. doi:10.1128/JVI.00176-10.

[133] L. Miorin, I. Romero-Brey, P. Maiuri, S. Hoppe, J. Krijnse-Locker, R.

Bartenschlager, et al., Three-Dimensional Architecture of Tick-Borne Encephalitis Virus Replication Sites and Trafficking of the Replicated RNA, J. Virol. 87 (2013) 6469–6481. doi:10.1128/JVI.03456-12.

[134] K.J. Lubick, S.J. Robertson, K.L. McNally, B.A. Freedman, A.L. Rasmussen, R.T. Taylor, et al., Flavivirus Antagonism of Type I Interferon Signaling Reveals Prolidase as a Regulator of IFNAR1 Surface Expression., Cell Host Microbe. 18 (2015) 61–74. doi:10.1016/j.chom.2015.06.007.

[135] S.M. Best, K.L. Morris, J.G. Shannon, S.J. Robertson, D.N. Mitzel, G.S. Park, et al., Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist., J. Virol. 79 (2005) 12828–12839. doi:10.1128/JVI.79.20.12828-12839.2005.

[136] K. Werme, M. Wigerius, M. Johansson, Tick-borne encephalitis virus NS5 associates with membrane protein scribble and impairs interferon-stimulated JAK-STAT signalling., Cell. Microbiol. 10 (2008) 696–712. doi:10.1111/j.1462-5822.2007.01076.x.

[137] R. Taylor, K. Lubick, S. Robertson, J. Broughton, TRIM79α, an interferon-stimulated gene product, restricts tick-borne encephalitis virus replication by degrading the viral RNA polymerase, Cell Host. (2011). http://www.sciencedirect.com/science/article/pii/S193131281100254X (accessed August 10, 2016).

[138] S.J. Robertson, K.J. Lubick, B. a Freedman, A.B. Carmody, S.M. Best, Tick-borne flaviviruses antagonize both IRF-1 and type I IFN signaling to inhibit dendritic cell function., J. Immunol. 192 (2014) 2744–55. doi:10.4049/jimmunol.1302110.

[139] T.-H. Chang, C.-L. Liao, Y.-L. Lin, Flavivirus induces interferon-beta gene expression through a pathway involving RIG-I-dependent IRF-3 and PI3K-dependent NF-κB activation, Microbes Infect. 8 (2006) 157–171. doi:10.1016/j.micinf.2005.06.014.

[140] S. Lienenklaus, M. Cornitescu, N. Zietara, M. Lyszkiewicz, N. Gekara, J. Jablonska, et al., Novel Reporter Mouse Reveals Constitutive and Inflammatory Expression of IFN- In Vivo, J. Immunol. 183 (2009) 3229–3236. doi:10.4049/jimmunol.0804277.

[141] M.E. Harper, L.M. Marselle, R.C. Gallo, F. Wong-Staal, Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization., Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 772–6. http://www.ncbi.nlm.nih.gov/pubmed/3003749 (accessed September 1, 2016).

[142] H.W. Pees, P.G. Scheurlen, Erkrankungen der Milz, Splenomegalie, in: Differ. Der Inn. Medizin, Springer Berlin Heidelberg, Berlin, Heidelberg, 1989: pp. 85–88.

doi:10.1007/978-3-642-73507-3_7.

[143] M. Cesta, Normal Structure, Function, and Histology of the Spleen, Toxicol. Pathol. 34 (2006) 455–465. doi:10.1080/01926230600867743.

[144] B.M. Dutia, C.J. Clarke, D.J. Allen, A.A. Nash, Pathological changes in the spleens of gamma interferon receptor-deficient mice infected with murine gammaherpesvirus: a role for CD8 T cells., J. Virol. 71 (1997) 4278–83. http://www.ncbi.nlm.nih.gov/pubmed/9151815 (accessed September 1, 2016).

[145] L. Kremer, J. Estaquier, I. Wolowczuk, F. Biet, J.C. Ameisen, C. Locht, Ineffective cellular immune response associated with T-cell apoptosis in susceptible Mycobacterium bovis BCG-infected mice., Infect. Immun. 68 (2000) 4264–73. http://www.ncbi.nlm.nih.gov/pubmed/10858244 (accessed September 21, 2016).

[146] F.K. Swirski, M. Nahrendorf, M. Etzrodt, M. Wildgruber, V. Cortez-Retamozo, P. Panizzi, et al., Identification of splenic reservoir monocytes and their deployment to inflammatory sites., Science. 325 (2009) 612–6. doi:10.1126/science.1175202.

[147] V. Cortez-Retamozo, M. Etzrodt, A. Newton, P.J. Rauch, A. Chudnovskiy, C. Berger, et al., Origins of tumor-associated macrophages and neutrophils, Proc. Natl. Acad. Sci. 109 (2012) 2491–2496. doi:10.1073/pnas.1113744109.

[148] B. Shrestha, M.S. Diamond, Role of CD8+ T Cells in Control of West Nile Virus Infection, J. Virol. 78 (2004) 8312–8321. doi:10.1128/JVI.78.15.8312-8321.2004.

[149] J. Hou, P. Wang, L. Lin, X. Liu, F. Ma, H. An, et al., MicroRNA-146a Feedback Inhibits RIG-I-Dependent Type I IFN Production in Macrophages by Targeting TRAF6, IRAK1, and IRAK2, J. Immunol. 183 (2009) 2150–2158. doi:10.4049/jimmunol.0900707.

[150] Y.-J. Liu, IPC: Professional Type 1 Interferon-Producing Cells and Plasmacytoid Dendritic Cell Precursors, Annu. Rev. Immunol. 23 (2005) 275–306. doi:10.1146/annurev.immunol.23.021704.115633.

[151] C. Kurhade, L. Zegenhagen, E. Weber, S. Nair, K. Michaelsen-Preusse, J. Spanier, et al., Type I Interferon response in olfactory bulb, the site of tick-borne flavivirus accumulation, is primarily regulated by IPS-1, J. Neuroinflammation. 13 (2016) 22. doi:10.1186/s12974-016-0487-9.

[152] H. Cho, S.C. Proll, K.J. Szretter, M.G. Katze, M. Gale, M.S. Diamond, Differential innate immune response programs in neuronal subtypes determine susceptibility to infection in the brain by positive-stranded RNA viruses., Nat. Med. 19 (2013) 1–8. doi:10.1038/nm.3108.

[153] J. Zhou, S.A. Stohlman, D.R. Hinton, N.W. Marten, Neutrophils Promote Mononuclear Cell Infiltration During Viral-Induced Encephalitis, J. Immunol. 170 (2003) 3331–3336. doi:10.4049/jimmunol.170.6.3331.

[154] G. Stollg, S. Jander, The role of microglia and macrophages in the pathophysiology of the CNS, Prog. Neurobiol. 58 (1999) 233–247. doi:10.1016/S0301-0082(98)00083-5.

[155] G.W. Kreutzberg, Microglia: a sensor for pathological events in the CNS, Trends Neurosci. 19 (1996) 312–318. doi:10.1016/0166-2236(96)10049-7.

[156] J.D. Sedgwick, S. Schwender, H. Imrich, R. Dorries, G.W. Butcher, V. ter Meulen, Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system., Proc. Natl. Acad. Sci. 88 (1991) 7438–7442. doi:10.1073/pnas.88.16.7438.

[157] A.N. van den Pol, S. Ding, M.D. Robek, Long-distance interferon signaling within the brain blocks virus spread., J. Virol. 88 (2014) 3695–704. doi:10.1128/JVI.03509-13.

[158] D. Van Antwerp, S. Martin, T. Kafri, D. Green, Suppression of TNF-alpha-induced apoptosis by NF-kB, Science (80-.). (1996). http://search.proquest.com/openview/2d601589ad63bba28679d1d154fb8a5c/1?pq-origsite=gscholar (accessed September 1, 2016).

[159] E. Gelpi, M. Preusser, F. Garzuly, H. Holzmann, F.X. Heinz, H. Budka, et al., Visualization of Central European tick-borne encephalitis infection in fatal human cases., J. Neuropathol. Exp. Neurol. 64 (2005) 506–12. doi:10.1093/jnen/64.6.506.

[160] K.D. Ryman, W.B. Klimstra, K.B. Nguyen, C.A. Biron, R.E. Johnston, Alpha/Beta Interferon Protects Adult Mice from Fatal Sindbis Virus Infection and Is an Important Determinant of Cell and Tissue Tropism, J. Virol. 74 (2000) 3366–3378. doi:10.1128/JVI.74.7.3366-3378.2000.

[161] M. Ida-Hosonuma, T. Iwasaki, T. Yoshikawa, N. Nagata, Y. Sato, T. Sata, et al., The Alpha/Beta Interferon Response Controls Tissue Tropism and Pathogenicity of Poliovirus, J. Virol. 79 (2005) 4460–4469. doi:10.1128/JVI.79.7.4460-4469.2005.

[162] D.-C. Lie, S. a Colamarino, H.-J. Song, L. Désiré, H. Mira, A. Consiglio, et al., Wnt signalling regulates adult hippocampal neurogenesis., Nature. 437 (2005) 1370–1375. doi:10.1038/nature04108.

[163] F. Aloisi, F. Ria, L. Adorini, Regulation of T-cell responses by CNS antigen-presenting cells: Different roles for microglia and astrocytes, Immunol. Today. 21 (2000) 141–148. doi:10.1016/S0167-5699(99)01512-1.

[164] C.N. Detje, T. Meyer, H. Schmidt, D. Kreuz, J.K. Rose, I. Bechmann, et al., Local type I IFN receptor signaling protects against virus spread within the central nervous system., J. Immunol. 182 (2009) 2297–304. doi:10.4049/jimmunol.0800596.

[165] C.N. Detje, S. Lienenklaus, C. Chhatbar, J. Spanier, C.K. Prajeeth, C. Soldner, et

al., Upon Intranasal Vesicular Stomatitis Virus Infection, Astrocytes in the Olfactory Bulb Are Important Interferon Beta Producers That Protect from Lethal Encephalitis, J. Virol. 89 (2015) 2731–2738. doi:10.1128/JVI.02044-14.

[166] K. Yoshida, F.H. Gage, Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes, 1991. doi:10.1016/0006-8993(91)90385-9.

[167] J.K. Roth-Cross, S.J. Bender, S.R. Weiss, Murine coronavirus mouse hepatitis virus is recognized by MDA5 and induces type I interferon in brain macrophages/microglia., J. Virol. 82 (2008) 9829–38. doi:10.1128/JVI.01199-08.

[168] J. Charles A Janeway, P. Travers, M. Walport, M.J. Shlomchik, The complement system and innate immunity, (2001).

[169] S. Delhaye, S. Paul, G. Blakqori, M. Minet, F. Weber, P. Staeheli, et al., Neurons produce type I interferon during viral encephalitis., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 7835–40. doi:10.1073/pnas.0602460103.

[170] L.G. Guidotti, F. V Chisari, Noncytolytic control of viral infections by the innate and adaptive immune response., Annu. Rev. Immunol. 19 (2001) 65–91. doi:10.1146/annurev.immunol.19.1.65.

[171] H. Cho, B. Shrestha, G.C. Sen, M.S. Diamond, A role for Ifit2 in restricting West Nile virus infection in the brain., J. Virol. 87 (2013) 8363–71. doi:10.1128/JVI.01097-13.

[172] L.S. Reinert, L. Harder, C.K. Holm, M.B. Iversen, K.A. Horan, F. Dagnæs-Hansen, et al., TLR3 deficiency renders astrocytes permissive to herpes simplex virus infection and facilitates establishment of CNS infection in mice., J. Clin. Invest. 122 (2012) 1368–76. doi:10.1172/JCI60893.

[173] K.J. Lubick, S.J. Robertson, K.L. McNally, B.A. Freedman, A.L. Rasmussen, R.T. Taylor, et al., Flavivirus Antagonism of Type I Interferon Signaling Reveals Prolidase as a Regulator of IFNAR1 Surface Expression, Cell Host Microbe. 18 (2015) 61–74. doi:10.1016/j.chom.2015.06.007.

[174] B.D. Rudd, J.J. Smit, R.A. Flavell, L. Alexopoulou, M.A. Schaller, A. Gruber, et al., Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection., J. Immunol. 176 (2006) 1937–42. doi:10.4049/JIMMUNOL.176.3.1937.

[175] C.W. Mandl, Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis, Virus Res. 111 (2005) 161–174. doi:10.1016/j.virusres.2005.04.007.

[176] S. Heinz, V. Haehnel, M. Karaghiosoff, L. Schwarzfischer, M. Müller, S.W. Krause, et al., Species-specific regulation of Toll-like receptor 3 genes in men and mice., J. Biol. Chem. 278 (2003) 21502–9. doi:10.1074/jbc.M301476200.

[177] M. Muzio, D. Bosisio, N. Polentarutti, G. D'amico, A. Stoppacciaro, R. Mancinelli, et al., Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells., J. Immunol. 164 (2000) 5998–6004. http://www.ncbi.nlm.nih.gov/pubmed/10820283 (accessed September 27, 2016).

[178] L. Alexopoulou, A.C. Holt, R. Medzhitov, R.A. Flavell, Recognition of double-stranded RNA and activation of NF-|[kappa]|B by Toll-like receptor 3, Nature. 413 (2001) 732–738. doi:10.1038/35099560.

[179] A.M. Lundberg, S.K. Drexler, C. Monaco, L.M. Williams, S.M. Sacre, M. Feldmann, et al., Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems, Blood. 110 (2007).

[180] A.K. Overby, V.L. Popov, M. Niedrig, F. Weber, Tick-Borne Encephalitis Virus Delays Interferon Induction and Hides Its Double-Stranded RNA in Intracellular Membrane Vesicles, J. Virol. 84 (2010) 8470–8483. doi:10.1128/JVI.00176-10.

[181] H. Harada, T. Taniguchi, N. Tanaka, The role of interferon regulatory factors in the interferon system and cell growth control, Biochimie. 80 (1998) 641–650. doi:10.1016/S0300-9084(99)80017-0.

[182] T. Krausgruber, D. Saliba, G. Ryzhakov, A. Lanfrancotti, IRF5 is required for late-phase TNF secretion by human dendritic cells, Blood. (2010). http://www.bloodjournal.org/content/115/22/4421.short (accessed September 21, 2016).

[183] B.J. Barnes, J. Richards, M. Mancl, S. Hanash, L. Beretta, P.M. Pitha, Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection., J. Biol. Chem. 279 (2004) 45194–207. doi:10.1074/jbc.M400726200.

[184] L.B. Thackray, B. Shrestha, J.M. Richner, J.J. Miner, A.K. Pinto, H.M. Lazear, et al., Interferon Regulatory Factor 5-Dependent Immune Responses in the Draining Lymph Node Protect against West Nile Virus Infection, J. Virol. 88 (2014) 11007–11021. doi:10.1128/JVI.01545-14.

[185] S. Daffis, M.A. Samuel, B.C. Keller, M. Gale, M.S. Diamond, Cell-specific IRF-3 responses protect against West Nile virus infection by interferon-dependent and -independent mechanisms., PLoS Pathog. 3 (2007) e106. doi:10.1371/journal.ppat.0030106.

[186] S. Costinean, N. Zanesi, Y. Pekarsky, E. Tili, S. Volinia, N. Heerema, et al., Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 7024–9. doi:10.1073/pnas.0602266103.

[187] H.M. Lazear, A. Lancaster, C. Wilkins, M.S. Suthar, A. Huang, S.C. Vick, et al.,

IRF-3, IRF-5, and IRF-7 Coordinately Regulate the Type I IFN Response in Myeloid Dendritic Cells Downstream of MAVS Signaling, PLoS Pathog. 9 (2013) e1003118. doi:10.1371/journal.ppat.1003118.

[188] M.J. Carson, J.M. Doose, B. Melchior, C.D. Schmid, C.C. Ploix, CNS immune privilege: Hiding in plain sight, Immunol. Rev. 213 (2006) 48–65. doi:10.1111/j.1600-065X.2006.00441.x.

[189] L.P. McCluskey, L.A. Lampson, Local immune regulation in the central nervous system by substance P vs. glutamate., J. Neuroimmunol. 116 (2001) 136–146. doi:10.1016/S0165-5728(01)00295-8.

[190] S. Nair, M.S. Diamond, Innate immune interactions within the central nervous system modulate pathogenesis of viral infections, Curr. Opin. Immunol. 36 (2015) 47–53. doi:10.1016/j.coi.2015.06.011.

[191] H.-M. Gao, J.-S. Hong, Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression, Trends Immunol. 29 (2008) 357–365. doi:10.1016/j.it.2008.05.002.

[192] C. Schwerk, T. Tenenbaum, K.S. Kim, H. Schroten, The choroid plexus-a multi-role player during infectious diseases of the CNS., Front. Cell. Neurosci. 9 (2015) 80. doi:10.3389/fncel.2015.00080.

[193] S.S. Ousman, P. Kubes, Immune surveillance in the central nervous system, Nat. Neurosci. 15 (2012) 1096–1101. doi:10.1038/nn.3161.

[194] T.O. Kleine, L. Benes, Immune surveillance of the human central nervous system (CNS): Different migration pathways of immune cells through theblood–brain barrier and blood–cerebrospinal fluid barrier in healthy persons, Cytom. Part A. 69A (2006) 147–151. doi:10.1002/cyto.a.20225.

[195] S. Fitting, S. Zou, W. Chen, P. Vo, K.F. Hauser, P.E. Knapp, Regional heterogeneity and diversity in cytokine and chemokine production by astroglia: differential responses to HIV-1 Tat, gp120, and morphine revealed by multiplex analysis., J. Proteome Res. 9 (2010) 1795–804. doi:10.1021/pr900926n.

[196] W.G. Kim, R.P. Mohney, B. Wilson, G.H. Jeohn, B. Liu, J.S. Hong, Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia., J. Neurosci. 20 (2000) 6309–6316. doi:20/16/6309 [pii].

[197] J.K. Roth-Cross, S.J. Bender, S.R. Weiss, Murine Coronavirus Mouse Hepatitis Virus Is Recognized by MDA5 and Induces Type I Interferon in Brain Macrophages/Microglia, J. Virol. 82 (2008) 9829–9838. doi:10.1128/JVI.01199-08.

[198] L. Schnell, S. Fearn, H. Klassen, M.E. Schwab, V.H. Perry, Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord., Eur. J. Neurosci. 11 (1999) 3648–3658. doi:10.1046/j.1460-

9568.1999.00792.x.

[199] K.L.W. Schultz, P.S. Vernon, D.E. Griffin, Differentiation of neurons restricts Arbovirus replication and increases expression of the alpha isoform of IRF-7., J. Virol. 89 (2015) 48–60. doi:10.1128/JVI.02394-14.

[200] D.M. Durrant, S. Ghosh, R.S. Klein, The Olfactory Bulb: An Immunosensory Effector Organ during Neurotropic Viral Infections, ACS Chem. Neurosci. 7 (2016) 464–469. doi:10.1021/acschemneuro.6b00043.

[201] E.M. Barnett, M.D. Cassell, S. Perlman, Two neurotropic viruses, herpes simplex virus type 1 and mouse hepatitis virus, spread along different neural pathways from the main olfactory bulb, Neuroscience. 57 (1993) 1007–1025. doi:10.1016/0306-4522(93)90045-H.

[202] M. Kipp, A. Norkute, S. Johann, L. Lorenz, A. Braun, A. Hieble, et al., Brain-region-specific astroglial responses in vitro after LPS exposure, J. Mol. Neurosci. 35 (2008) 235–243. doi:10.1007/s12031-008-9057-7.

[203] L.A. Mongiat, M.S. Espósito, G. Lombardi, A.F. Schinder, Reliable Activation of Immature Neurons in the Adult Hippocampus, PLoS One. 4 (2009) e5320. doi:10.1371/journal.pone.0005320.

[204] A. Carleton, L.T. Petreanu, R. Lansford, A. Alvarez-Buylla, P.-M. Lledo, Becoming a new neuron in the adult olfactory bulb, Nat. Neurosci. 6 (2003) 507. doi:10.1038/nn1048.

[205] K.M. Castorena, D.C. Peltier, W. Peng, D.J. Miller, Maturation-dependent responses of human neuronal cells to western equine encephalitis virus infection and type I interferons., Virology. 372 (2008) 208–20. doi:10.1016/j.virol.2007.10.025.

[206] T. Seki, Hippocampal adult neurogenesis occurs in a microenvironment provided by PSA-NCAM-expressing immature neurons, J. Neurosci. Res. 69 (2002) 772–783. doi:10.1002/jnr.10366.

[207] H. Neumann, I.M. Medana, J. Bauer, H. Lassmann, Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases, Trends Neurosci. 25 (2002) 313–319. doi:10.1016/S0166-2236(02)02154-9.

[208] S.L. Bailey, B. Schreiner, E.J. McMahon, S.D. Miller, CNS myeloid DCs presenting endogenous myelin peptides “preferentially” polarize CD4+ TH-17 cells in relapsing EAE, Nat. Immunol. 8 (2007) 172–180. doi:10.1038/ni1430.

[209] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8 (2008) 958–969. doi:10.1038/nri2448.

[210] L.T. Remington, A.A. Babcock, S.P. Zehntner, T. Owens, Microglial Recruitment, Activation, and Proliferation in Response to Primary Demyelination, Am. J. Pathol.

170 (2007) 1713–1724. doi:10.2353/ajpath.2007.060783.

[211] G. Reichmann, M. Schroeter, S. Jander, H.-G. Fischer, Dendritic cells and dendritic-like microglia in focal cortical ischemia of the mouse brain, J. Neuroimmunol. 129 (2002) 125–132. doi:10.1016/S0165-5728(02)00184-4.

[212] N. Hagemeyer, M. Prinz, Burning down the house: IRF7 makes the difference for microglia., EMBO J. 33 (2014) 2885–6. doi:10.15252/embj.201490345.

[213] Z. Hongmei, Extrinsic and Intrinsic Apoptosis Signal Pathway Review, in: Apoptosis Med., InTech, 2012. doi:10.5772/50129.

[214] S.W. Dow, M.J. Dreitz, E.A. Hoover, Feline immunodeficiency virus neurotropism: evidence that astrocytes and microglia are the primary target cells, Vet. Immunol. Immunopathol. 35 (1992) 23–35. doi:10.1016/0165-2427(92)90118-A.

[215] S. Perlmans, D. Ries, The astrocyte is a target cell in mice persistently infected with mouse hepatitis virus, strain JHM, Academic Press, 1987. doi:10.1016/0882-4010(87)90064-7.

[216] B. Melchior, S.S. Puntambekar, M.J. Carson, Microglia and the control of autoreactive T cell responses., Neurochem. Int. 49 (2006) 145–53. doi:10.1016/j.neuint.2006.04.002.

[217] M.A. Samuel, M.S. Diamond, Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival., J. Virol. 79 (2005) 13350–61. doi:10.1128/JVI.79.21.13350-13361.2005.

[218] J.J. Gao, M.B. Filla, M.J. Fultz, S.N. Vogel, S.W. Russell, W.J. Murphy, Autocrine/paracrine IFN-alphabeta mediates the lipopolysaccharide-induced activation of transcription factor Stat1alpha in mouse macrophages: pivotal role of Stat1alpha in induction of the inducible nitric oxide synthase gene., J. Immunol. 161 (1998) 4803–10. http://www.ncbi.nlm.nih.gov/pubmed/9794412 (accessed September 21, 2016).

[219] F.X. Zhu, S.M. King, E.J. Smith, D.E. Levy, Y. Yuan, A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation., Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 5573–8. doi:10.1073/pnas.082420599.

[220] C.F. Basler, X. Wang, E. Mühlberger, V. Volchkov, J. Paragas, H.D. Klenk, et al., The Ebola virus VP35 protein functions as a type I IFN antagonist., Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12289–94. doi:10.1073/pnas.220398297.

[221] N.K. Duggal, M. Emerman, Evolutionary conflicts between viruses and restriction factors shape immunity, Nat. Rev. Immunol. 12 (2012) 687–695. doi:10.1038/nri3295.

[222] R.A. Lukaszewski, T.J. Brooks, Pegylated alpha interferon is an effective treatment

for virulent venezuelan equine encephalitis virus and has profound effects on the host immune response to infection., J. Virol. 74 (2000) 5006–15. doi:10.1128/JVI.74.11.5006-5015.2000.

[223] T.I.M.S.S. Group, Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group., Neurology. 43 (1993) 655–61. doi:10.1212/WNL.43.4.655.

[224] M. Cohen, O. Matcovitch, E. David, Z. Barnett-Itzhaki, H. Keren-Shaul, R. Blecher-Gonen, et al., Chronic exposure to TGFβ1 regulates myeloid cell inflammatory response in an IRF7-dependent manner., EMBO J. 33 (2014) 2906–21. doi:10.15252/embj.201489293.

[225] E. Cassol, L. Cassetta, C. Rizzi, M. Alfano, G. Poli, M1 and M2a polarization of human monocyte-derived macrophages inhibits HIV-1 replication by distinct mechanisms., J. Immunol. 182 (2009) 6237–46. doi:10.4049/jimmunol.0803447.

[226] V.E. Miron, A. Boyd, J.-W. Zhao, T.J. Yuen, J.M. Ruckh, J.L. Shadrach, et al., M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination, Nat. Neurosci. 16 (2013) 1211–1218. doi:10.1038/nn.3469.

[227] L. Durelli, L. Conti, M. Clerico, D. Boselli, G. Contessa, P. Ripellino, et al., T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-β, Ann. Neurol. 65 (2009) 499–509. doi:10.1002/ana.21652.

[228] K.-P. Wandinger, C.-S. Stürzebecher, B. Bielekova, G. Detore, A. Rosenwald, L.M. Staudt, et al., Complex immunomodulatory effects of interferon-β in multiple sclerosis include the upregulation of T helper 1-associated marker genes, Ann. Neurol. 50 (2001) 349–357. doi:10.1002/ana.1096.

[229] M. Haglund, G. Günther, Tick-borne encephalitis—pathogenesis, clinical course and long-term follow-up, Vaccine. 21 (2003) S11–S18. doi:10.1016/S0264-410X(02)00811-3.

[230] J.F. Annegers, W.A. Hauser, E. Beghi, A. Nicolosi, L.T. Kurland, The risk of unprovoked seizures after encephalitis and meningitis., Neurology. 38 (1988) 1407–10. doi:10.1212/WNL.38.9.1407.

[231] N. McGrath, N.E. Anderson, M.C. Croxson, K.F. Powell, Herpes simplex encephalitis treated with acyclovir: diagnosis and long term outcome., J. Neurol. Neurosurg. Psychiatry. 63 (1997) 321–6. doi:10.1136/JNNP.63.3.321.

[232] T. Solomon, N.M. Dung, B. Wills, R. Kneen, M. Gainsborough, T.V. Diet, et al., Interferon alfa-2a in Japanese encephalitis: a randomised double-blind placebo-controlled trial, Lancet. 361 (2003) 821–826. doi:10.1016/S0140-6736(03)12709-2.

[233] R. Nandakumar, K. Finsterbusch, C. Lipps, B. Neumann, M. Grashoff, S. Nair, et

al., Hepatitis C Virus Replication in Mouse Cells Is Restricted by IFN-Dependent and -Independent Mechanisms, Gastroenterology. 145 (2013) 1414–1423.e1. doi:10.1053/j.gastro.2013.08.037.

[234] J. Schwerk, M. Köster, H. Hauser, M. Rohde, M. Fulde, M.W. Hornef, et al., Generation of Mouse Small Intestinal Epithelial Cell Lines That Allow the Analysis of Specific Innate Immune Functions, PLoS One. 8 (2013) e72700. doi:10.1371/journal.pone.0072700.

[235] G.-P. Shi, J.A. Villadangos, G. Dranoff, C. Small, L. Gu, K.J. Haley, et al., Cathepsin S Required for Normal MHC Class II Peptide Loading and Germinal Center Development, Immunity. 10 (1999) 197–206. doi:10.1016/S1074-7613(00)80020-5.

[236] M. Niedrig, U. Klockmann, W. Lang, J. Roeder, S. Burk, S. Modrow, et al., Monoclonal antibodies directed against tick-borne encephalitis virus with neutralizing activity in vivo., Acta Virol. 38 (1994) 141–149.

[237] D.C. Rio, M. Ares, G.J. Hannon, T.W. Nilsen, Purification of RNA Using TRIzol (TRI Reagent), Cold Spring Harb. Protoc. 2010 (2010) pdb.prot5439–pdb.prot5439. doi:10.1101/pdb.prot5439.

6. Appendix

Abbreviations

au	arbitrary units
BBB	blood brain barrier
BSA	bovine serum albumin
BSL	biosafety level
BMDCs	bone marrow derived cells
BMDM	bone marrow derived macrophages
°C	degree Celsius
CCL	Chemokine (C-C motif) ligand
CCr	C-C chemokine receptor type
CD	cluster of differentiation
CLRs	C-type lectin receptors
cm	centimeter
CNS	central nervous system
CP	crossing point
CSFB	cerebrospinal fluid *barrier*
CXCL	chemokine (C-X-C motif) ligand
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DCs	dendritic cells
DENV	dengue virus
dpi	days post infection
cAMP	Cyclic adenosine monophosphate
CREB	cAMP responsive element binding protein
EMCV	Encephalomyocarditis virus
ER	endoplasmatic reticulum
FCS	fetal calf serum
FFU	Focus forming units
g	gram
GAS	gamma-IFN activated sites
GC	granulocytes
Hpi	hours post infection
Hpt	hours post transfection
HSV	Herpes simplex virus

IAD	IRF associated domains
i.c.	intra cranial
IFN-β/α	Interferon alpha/beta
IFNAR	IFN-α/β receptor
IFNGR	IFN-γ gamma receptor
IHC	immunhistochemistry
IKKε	IkB kinase ε
IL	Interleukin
i.p.	intra peritoneal
IPCs	IFN producing cells
IPS-1	IFN-β promotor stimulator -1
IRF	interferon regulatory factor
ISGF3	IFN-stimulated gene factor 3
ISGs	IFN stimulated genes
ISRE	IFN stimulated response element
Jak	Janus kinase
JeV	Japanese encephalitis virus
kDa	kilodalton
LGTV	Langat virus
LN	lymph node
Luc	luciferase
MDA5	Melanoma differentiation associated protein 5
mDCs	myeloid DCs
MHC	major histocompatibility complex
ml	milliliter
µm	micrometer
MOI	multiplicity of infection
mTOR	mechanistic Target of Rapamycin
Myd88	Myeloid differentiation primary response gene 88
MZ	marginal zone
Nalt	nasal associated lymphoid tissue
NDV	Newcastle disease virus
NETs	neutrophil extracellular trap
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
Nk cells	Natural killer cells
NLRs	NOD-like receptors
Nm	nanometer

NOD	nucleotide-binding oligomerization domain
NS	non-structural protein
OAS	2'-5'-oligoadenylate synthetase
OHFV	Omsk hemorrhagic fever virus
PAMPs	Pathogen associated molecular patterns
pDCs	plasmacytoid DCs
PKR	Protein kinase R
Poly I:C	polyinosinic-polycytidylic acid
POWV	Powassan virus
PRRs	pathogen recognition receptors
qRT-PCR	quantitative real time polymerase chain reaction
RIG-I	Retinoic acid inducible gene-I
RLU	relative light units
RLRs	retinoic acid–inducible gene-I (RIG-I)-like receptors
RMS	rostral migratory stream
RNA	ribonucleic acid
RP	red pulp
RT	room temperature
SCID	severe combined immunodeficiency
SEM	standard error of mean
SeV	Sendai virus
Stat	signal transducers and activators of transcription 1
TANK	TRAF family member-associated NF-kappa-B activator
TBEV	tick-borne encephalitis virus
TBK-1	TANK binding kinase-1
TGF-β	transforming growth factor-beta
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TRAF3	TNF receptor associated factor 3
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM	tripartite motif
VSV	Vesicular stomatitis virus
WNV	West Nile virus
WP	white pulb
WT	wild type
ZIKV	Zika virus

Figure Legend

Table Legend

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Curriculum vitae

Personal information

Name:	Loreen Zegenhagen
E-mail:	loze04@web.de
Mobile:	0151/20460055
Nationality:	German
Date of birth:	30.06.1988, Cottbus

Study:

10/2013 –10/2016	**PhD Thesis** Helmholtz Centre for Infection Research, Prof. Dr. A. Kröger
10/2011– 09/2013	„**Master of Science**“ (Biology) TU-Braunschweig Focus: Infection biology and Cell biology
08/2011 – 07/2012	**Student assistant in research** TU-Braunschweig, Dr. S.Wöhl-Bruhn
09/2010 – 12/2010	**Erasmus Placement** Umeå University Sweden, Prof. Dr. S. Bergström
10/2008 – 09/2011	„**Bachelor of Science**“ (Biology) TU-Braunschweig Focus: Biochemistry and Genetics

Publications

“Type I Interferon response in olfactory bulb, the site of tick-borne flavivirus accumulation, is primarily regulated by IPS-1”; C.Kurhade, L.Zegenhagen, et al., 2016, Journal of Neuroinflammation

„Brain heterogeneity leads to differential innate immune responses and modulates pathogenesis of viral infections”; L.Zegenhagen et al., 2016, Cytokine and Growth Factor Reviews

„Differences in IPS-1-Mediated Innate Immune Responses between Neurotrophic Flavivirus Infection”; L.Zegenhagen et al., 2016, Journal of Neuroinfectious Diseases